INHIBITION OF REGENERATION IN THE INJURED, ADULT SPINAL CORD: POTENTIAL ROLE OF CHONDROITIN SULFATE PROTEOGLYCANS, SPECIFICALLY AGGRECAN

By

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This dissertation is dedicated to my family

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School
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Extrinsic factors appear to contribute to the lack of regeneration in the injured adult spinal cord. It is likely that these extrinsic factors include a group of putative growth inhibitory molecules known as the chondroitin sulfate proteoglycans (CSPGs). CSPGs are present in the spinal cord matrix and consist of a core protein with one or more chondroitin sulfate (CS) glycosaminoglycan (GAG) side chains. Studies in this dissertation show an increase in the immunoreactivity (IR) patterns of these putative growth inhibitory molecules following spinal cord injury as early as four and as late as forty days post-injury (the earliest and last time point examined) using immunohistochemistry techniques. CSPG-IR patterns are similar to glial fibrillary acidic protein (GFAP)-IR, suggesting that astrocytes may express CSPGs in the central nervous system (CNS). The increases in CSPG-IR following spinal cord injury are

correlated with a lack of regeneration in this system. These correlations support CSPGs' hypothesized inhibitory growth influences in the injured spinal cord.

These initial studies led us to examine the effects of CSPGs upon axonal growth *in vivo*. Scientific literature at this time suggested that the members of the CSPG family have different effects upon growth. In light of these studies, subsequent work focused on the effects of a specific CSPG, aggrecan. Using Western gel analysis, we identified aggrecan in the embryonic, postnatal, adult and injured spinal cord. Furthermore, our results also show that aggrecan can inhibit axonal growth in a novel microsurgical model referred to as an *in vivo* growth assay. Results from these studies also show that the core protein of aggrecan significantly inhibits growth compared to positive controls, although not as robustly as intact aggrecan. There is a trend for the CS GAG chains of aggrecan to mildly inhibit growth, but the difference in growth patterns is not always statistically significant from positive controls. Together, these results indicate that aggrecan can inhibit axonal growth *in vivo* and the presumed inhibitory component may primarily lie in the core protein of aggrecan. These studies suggest that aggrecan, a specific growth inhibitory CSPG, is present in the spinal cord and may contribute to the lack of regeneration following injury.

CHAPTER 1
BACKGROUND

The Central Nervous System's Failure to Regenerate

The mature, mammalian central nervous system (CNS) fails to successfully regenerate following injury. A classic anatomical hallmark of regenerative failure, the retraction bulb, was described in the early 1900's by Santiago Ramon y Cajal (Ramon y Cajal, 1991). Retraction bulbs are swollen endings at the central stumps of injured, degenerating axons. Cajal hypothesized that retraction bulbs resulted from an axon's progressive withdrawal and separation from its distal necrotic segment. He viewed a retraction bulb as "an act of sedentary reaction" by an axon with no energy to form new paths or overcome obstacles (Ramon y Cajal, 1991). Cajal also noted the lack of fibers in neuroglial tissue that formed following CNS injury. He hypothesized that this neuroglial tissue may be one of the obstacles that prevents an injured CNS neuron from regrowing it severed distal end. More recently, this neuroglial tissue has been described in greater detail as the glial scar that consists of hypertrophied reactive astrocytes which often appear to encapsulate a lesion (Houle, Reier, 1988; Reier et al., 1988b). This wall of reactive astrocytes may serve as a chemical and/or physical barrier to regrowth (Houle, 1992; Houle, Reier, 1988; Reier et al., 1988b). The inability of neurons to regenerate in the CNS can stem from: 1) extrinsic factors and/or 2) the intrinsic capacity of the neuron. It is impressive that nearly a century ago, Cajal described both extrinsic factors (neuroglial tissue) and intrinsic factors (the

formation of a retraction bulb by some neurons rather than a new process) that may contribute to regeneration failure by the way of fundamental light microscopy. More recent, technologically sophisticated studies, support Cajal's general hypotheses and suggest that both intrinsic and extrinsic factors can contribute to the failure of the CNS to regenerate.

In the late 1970's and early 1980's, the significance of extrinsic cues as well as the intrinsic capacity of some CNS neurons to grow long processes was illustrated using a peripheral nerve graft (Benfey, Aguayo, 1982; Richardson et al., 1984; So, Aguayo, 1985). A sciatic nerve grafted into the brainstem or the spinal cord appears to serve as a conduit or a bridge and support the growth of some CNS neurons for long distances (Andres et al., 1992). Although the distance of CNS axonal growth was impressive (up to ~3.5cm), the number of spinal cord axons in the peripheral nerve graft was limited (Benfey, Aguayo, 1982; Richardson et al., 1984; So, Aguayo, 1985). The limited number of growing spinal cord axons suggests that some, but not all, neurons may have the intrinsic capacity to extend and maintain long processes. Interestingly, the neurons that did extend long processes through the graft, traveled only short distances (~2mm) upon re-entry into the spinal cord (Aguayo et al., 1981). These results suggest that the CNS environment may limit the growth of some spinal cord neurons that have the intrinsic capacity to extend and maintain long processes. Thus, extrinsic/environmental cues appear to play an important role in the success or failure of regeneration. The failure of the spinal cord environment to promote growth may be due to several factors including: 1) the lack of sufficient growth promoting molecules and/or 2) the presence of significant growth inhibitory molecules. Based upon this reasoning, axonal growth may be enhanced

by: 1) addition of growth promoting molecules and/or 2) removal of growth inhibitory molecules. The former has led many investigators to modify the spinal cord terrain with the addition of growth promoting molecules/tissue in hopes of promoting growth.

Attempts to Promote Regeneration

A variety of approaches have been used to promote regeneration in the spinal cord. Some of the earliest studies that enhanced axonal growth used intraspinal transplantation of embryonic tissue. Grafts of embryonic tissue from a variety of CNS sources were shown to promote some axonal growth in the injured adult spinal cord (Anderson et al., 1995a; Anderson et al., 1995b; Bernstein, Bregman, 1993; Howland et al., 1996; Itoh, Tessler, 1990; Jakeman, Reier, 1991; Reier et al., 1986; Tessler et al., 1988). The extent of growth into embryonic transplants (determined by immunohistochemistry and neuroanatomical tracing techniques) can be enhanced by exogenous application of neurotrophins to the transplant site. For example, application of brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or neurotrophin-4 (NT-4) appears to promote greater axonal growth from the host into neurotrophin-treated transplants compared to vehicle-treated transplants (Bregman et al., 1997). Furthermore, use of select neurotrophins alone have also been used to enhance host axonal growth. Neurotrophins can enhance axonal growth when applied exogenously or when secreted by genetically engineered cells. For example, exogenous application of either BDNF or NT-3 promotes increased axonal growth into Schwann cell impregnanted channels compared to non-treated Schwann cell containing channels (Xu et al., 1995). In addition, transplantation of genetically engineered cells, such as fibroblasts, that secrete nerve

growth factor, NT-3, BDNF or ciliary neurotrophic factor promotes enhanced growth in the spinal cord (Nakahara et al., 1996; Tuszynski et al., 1994). These studies suggest that embryonic tissue and/or addition of neurotrophins can promote some axonal growth in the spinal cord. However, these techniques do not result in complete regeneration. It is likely that other extrinsic factors, such as growth inhibitory molecules, may be preventing full regeneration in the injured spinal cord, even in the presence of growth promoting molecules.

Molecules that May Inhibit Regeneration

Some specific molecules in the spinal cord have been identified that appear to inhibit growth. For example, myelin from mammalian CNS appears to inhibit neurite growth of rat sympathetic neurons, mouse 3T3 cells and mouse neuroblastoma cells *in vitro* (Caroni, Schwab, 1988b; Schwab, Caroni, 1988). Specifically, two myelin proteins (35 and 250kD) have been identified as growth inhibitory components of myelin (Caroni, Schwab, 1988b). Their apparent growth inhibitory effects can be neutralized *in vitro* by antibodies (IN-1 and IN-2) that were specifically raised against these myelin proteins (Caroni, Schwab, 1988a). Furthermore, application of IN-1 antibodies appear to partially neutralize the inhibitory effect of CNS white matter upon the adhesion of neuroblastoma cells seeded on cryosections of mammalian CNS tissue (Savio, Schwab, 1989). Application of IN-1 antibodies *in vivo* also promotes greater axonal growth in the injured spinal cord compared to controls (Bregman et al., 1995; Schnell, Schwab, 1990). These studies suggest that myelin proteins may contribute to the lack of successful regeneration following spinal cord injury.

Recent reports, however, challenge the putative growth inhibitory influence of these myelin proteins. Microtransplanted embryonic neurons (from a variety of sources including hippocampus and dorsal root ganglion) can extend long processes in adult white matter tracts (including fimbria and spinal cord funiculus} *in vivo* (Davies et al., 1994; Davies et al., 1997; Davies et al., 1993; Davies et al., 1994). The ability of these transplanted embryonic neurons to grow processes within adult white matter tracts suggests that myelin can be permissive to these neurons in this minimally-traumatic *in vivo* model. Adult and neonatal neurons from the dorsal root ganglion can also extend long processes when microtransplanted into the adult corpus callosum (Davies et al., 1997). However, some of these transplants did not extend long processes. In these instances, the graft was surrounded by chondroitin sulfate proteoglycans (CSPGs) (Davies et al., 1997). This correlation of regeneration failure with the presence of CSPGs suggests that these molecules may have the capacity to inhibit regeneration. This was not the first study to suggest that CSPGs have growth inhibitory influences. The potential growth inhibitory nature of CSPGs is also suggested by: 1) their growth inhibitory effects in cell culture assays (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Dou, Levine, 1995; Perris, Johansson, 1987; Rudge, Silver, 1990; Snow, Letourneau, 1992; Yamada et al., 1997), 2) their presence in boundary tissues during development (Fichard et al., 1991; Landolt et al., 1995; Oakley et al., 1994; Oakley, Tosney, 1991; Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990) and 3) their spatial and temporal correlation with the lack of regeneration in the injured spinal cord (Davies et al., 1997; Fitch, Silver, 1997; Gates et al., 1996; Lemons et al., 1999). These studies are discussed in more detail below.

Evidence for CSPGs as Growth Inhibitory Molecules

CSPGs are putative growth inhibitory molecules that consist of a core protein with one or more chondroitin sulfate glycosaminoglycan (GAG) chains. A GAG chain is an alternating, linear, unbranched sequence of disaccharides. For example, a CS GAG chain consists of alternating patterns on N-acetylgalactosamine and glucuronic acid (Kjellen, Lindahl, 1991b; Roughley, Lee, 1994; Ruoslahti, 1988; Wight et al., 1991). The putative inhibitory nature of CSPGs was initially suggested by developmental studies.

CSPGs Appear to Be Repulsive During Development

During development, CSPGs appear to form boundaries that repel growing axons and thus, help guide them to their appropriate targets (Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990; Tosney, Oakley, 1990). Once the appropriate targets are innervated, CSPG-immunoreactivity (IR) increases around the connected axons. This increase in CSPG-IR is spatially and temporally correlated with a lack of any further growth (Dow et al., 1994; Pindzola et al., 1991; Steindler, 1993). For example, by postnatal day three in the developing spinal cord, the distribution of CSPGs spreads laterally from the midline to include the dorsal root entry zone. The onset of CSPG expression in the dorsal root entry zone coincides with the end of the period of axonal growth into and through this area (Dow et al., 1994; Pindzola et al., 1991).

In addition, migrating neural crest cells avoid CSPG positive areas such as the perinotochordal mesenchyme and subepidermal spaces during periods of cell movement and axonal elongation (Oakley et al., 1994; Oakley, Tosney, 1991; Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990; Tosney, Oakley, 1990). The growth inhibiting

hypothesis drawn from *in vivo* correlational development studies is supported by many *in vitro* studies that show immature neurites avoid CSPG positive areas (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Katoh-Semba et al., 1995; Oohira et al., 1994; Perris, Johansson, 1987; Snow, Letourneau, 1992; Snow et al., 1991; Yamagata et al., 1989). CSPGs, therefore, appear to act as developmental guidance molecules by inhibiting neurite growth into inappropriate areas and blocking further growth into appropriate areas once targets have been sufficiently innervated.

Chondroitinase ABC Digestion Can Diminish Inhibition

The capacity of CSPGs to be repulsive during development appears to be diminished in some cases by treatment with a bacterial enzyme, chondroitinase ABC. Chondroitinase ABC cleaves chondroitin sulfate GAG chains on CSPGs while leaving the core protein intact with short sugar stubs. This enzymatic degradation can alter CSPGs' influence upon neurite growth, axonal guidance and cell migration. For example, *in vitro*, neurite growth from fetal dorsal root ganglion (DRG) cells plated on embryonic day 17 chick spinal cord extract is significantly greater after treatment with chondroitinase ABC (Dow et al., 1994). Cleavage of CSPGs with chondroitinase ABC disrupts CSPGs' barrier functions and results in disorganized axonal growth of embryonic retinal ganglion cells in an *in vitro* explant assay (Brittis et al., 1992). Cleavage of CSPGs also affects cell migration *in vivo*. Avian neural crest cells extend towards previously avoided CSPG positive notochord tissue *in vivo* following chondroitinase digestion (Brittis et al., 1992; Newgreen et al., 1986; Pettway et al., 1990). Thus, cleavage of CSPGs by chondroitinase ABC appears to disrupt CSPGs' influences upon neurite growth and cell migration which

suggests that intact GAG chains are required for CSPGs' inhibitory effects. These studies also further confirm CSPGs' roles as growth inhibitors. However, chondroitinase ABC digestion is not always a sufficient method to diminish CSPGs' growth inhibitory effects. In particular, one study has documented the ability of some CSPGs to continue to inhibit growth following chondroitinase ABC digestion (Dou, Levine, 1994). This discrepancy is discussed later in this chapter.

CSPGs Are Present in the Glial Scar

The glial scar that often forms following injury to the adult CNS has been traditionally regarded as an impediment to regeneration (Houle, Reier, 1988; Reier et al., 1988b). However its influences and the mechanisms by which it may inhibit growth are not well understood (Reier et al., 1989). Interestingly, there are both growth promoting (e.g., laminin) (Alonso, Privat, 1993; Frisén et al., 1995; McKeon et al., 1991; Risling et al., 1993) and growth inhibitory molecules (e.g., CSPGs) (McKeon et al., 1991; Risling et al., 1993) in the glial scar. It is possible that the lack of growth in the glial scar may be, at least in part, due to the presence of CSPGs (McKeon et al., 1991; Risling et al., 1993). CSPGs in the glial scar appear to have the ability to inhibit growth *in vitro* (Risling et al., 1993). The growth of retinal ganglion cells plated on explanted gliotic scar tissue increases following chondroitinase ABC digestion in culture. However, this increase in neuritic growth can be reversed by application of antibodies against laminin (Risling et al., 1993). A similar trend has been reported in cryocultured slices of spinal cord tissue (Zuo et al., 1998). Thus, CSPGs may limit or mask the growth promoting effects of laminin in the glial scar and may contribute to the lack of axonal growth through this tissue.

The growth inhibiting effects of CSPGs and the growth promoting effects of laminin do not appear to work in isolation, rather they appear to have an interactive relationship. The ability of CSPGs to mask or minimize laminin's growth promoting effects, although not well understood, may account for the relative lack of axons in the glial scar. *In vitro*, neurite growth is dependent upon the ratio of CSPGs to laminin (Snow, Letourneau, 1992). A low CSPG/high laminin substrate is known to promote cellular migration and neuritic growth whereas a high CSPG/low laminin substrate inhibits migration and growth. Thus, a high ratio of CSPGs to laminin in scar tissue may contribute to the limited neuronal growth into or through the glial scar.

Conflicting Evidence Regarding CSPGs' Influences

The putative growth inhibitory influences of CSPGs, like the myelin proteins, are disputed (Bicknese et al., 1994; Faissner et al., 1994; Gates et al., 1996; Iijima et al., 1991). There are some studies which suggest that CSPGs are growth facilitatory (Dow et al., 1993; Faissner et al., 1994; Gates et al., 1996; Iijima et al., 1991) and can have neurotrophic effects (Koops et al., 1996). These apparent contrasting effects of CSPGs may stem from methodological differences across several studies. In addition, these methodological differences may not only contribute to conflicting reports on CSPGs' influences, they may also provide information about possible mechanisms of CSPGs' influences. Some of the methodological differences include: source of the neurons, cell culture systems, and concentration and presentation of CSPGs. For example, the growth of neurons from different sources (including embryonic chick dorsal root ganglion,

embryonic chick retinal ganglion or embryonic rat forebrain) is not equally inhibited by CSPGs (Snow, Letourneau, 1992). Although these neurons from different sources are generally inhibited by CSPGs, they each behave differently in response to increasing concentrations of CSPGs (Snow, Letourneau, 1992). Interestingly, some cell culture studies that present CSPGs as growth permissive use embryonic neurons from a variety of different sources including the hippocampus, mesencephalon, and neocortex (Faissner et al., 1994; Junghans et al., 1995). The variety of neurons used in cell culture assays may contribute to some of the diversity of CSPGs' influences upon axonal growth. It is possible that CSPGs may influence distinct neurons differently.

Just as the source of a neuron is critical to data interpretation, so are the presentation and concentration of CSPGs. CSPGs that are cell-surface bound or in the ECM may function differently than secreted (soluble) CSPGs. For example, studies by Snow et al., (1996) demonstrate that substratum bound CSPGs inhibit neurite outgrowth on laminin in a concentration dependent manner whereas soluble CSPGs do not (Snow et al., 1996). In addition, the ability of bound CSPGs to inhibit growth can be overwhelmed by increasing concentrations of laminin (Snow, Letourneau, 1992), suggesting that the concentration of CSPGs (relative to laminin) used in cell culture studies may contribute to the appearance of CSPGs as being either growth promoting or growth inhibitory. Interestingly, the degree of neurite inhibition differs when CSPGs are bound to laminin compared to fibronectin substrates. CSPGs bound to laminin (100μg/ml) results in a 40% reduction in the number of neurons bearing neurites whereas CSPG bound to fibronectin (100μg/ml) results in an 80% reduction (Snow et al., 1996). These results illustrate the different

influences of distinct substrates commonly used in cell culture assays (e.g., laminin and fibronectin) upon CSPGs' ability to inhibit growth. In combination, these studies suggest that the growth inhibitory effects of CSPGs *in vitro* can be influenced by a variety of other extrinsic factors that often differ between cell culture studies. It is likely that the potentially wide variety of methodological differences, such as the source of neurons, presentation of CSPGs, and cell culture models, are likely to contribute to the conflicting reports of CSPG influences upon growth. Methodological differences not withstanding, the diversity of proteoglycans within the CSPG family is also likely to contribute to discrepancies in CSPGs' influences.

Diversity of CSPGs

Recently, specific proteoglycans have been identified and named by their unique core protein sequences. Many of these proteoglycans belong to the CSPG family and include: biglycan (Neame et al., 1989); decorin (Abramson, Woessner, 1992; Krusius, Ruoslahti, 1986), brevican (Seidenbecher et al., 1995; Yamada et al., 1994), NG2 (Dou, Levine, 1994; Levine, 1994), versican (Zimmermann, Ruoslahti, 1989), neurocan (Rauch et al., 1992) and aggrecan (Doege et al., 1991).

Each CSPG is distinct in several ways including: the protein core sequence, temporal and spatial expression patterns, and influences upon growth. For example, the sequences of specific CSPG core proteins reveal that some CSPGs are integral membrane proteins (e.g., NG2)(Burg et al., 1997; Nishiyama et al., 1991) whereas others are cell surface glycosylphosphatidylinositol-anchored proteins (e.g., brevican) (Seidenbecher et al.,

1995). The ability of some proteoglycans to attach to a cell surface may affect a CSPG's presentation to a neuron and thus, influence a CSPG's impact upon neurite growth. In addition, spatial and temporal expression patterns are unique for each CSPG and may also reflect each CSPG's distinct function or influence. For example, NG2 is present on glial precursor cells (Stallcup, Beasley, 1987) and in a variety of CNS and non-CNS tissues whereas neurocan is primarily expressed by neurons and is restricted to the nervous system (Engel et al., 1996).

The temporal expression patterns of distinct CSPGs are as unique as their spatial patterns. During development, traces of brevican are detected in embryonic brain and steadily increase after birth, whereas neurocan expression peaks in the early postnatal period and declines thereafter (Milev et al., 1998b). These distinct spatial and temporal patterns, in combination with their unique presentations *in vivo,* suggest that distinct CSPGs may have different functions. This hypothesis has been supported by several cell culture studies that report specific CSPGs have unique effects upon neurite growth. For example, DSD-1 (a CSPG that has not yet been sequenced) has been reported to promote neurite outgrowth (Faissner et al., 1994) whereas NG2 and brevican have been shown to inhibit neurite growth *in vitro* (Dou, Levine, 1994; Yamada et al., 1997). Furthermore, the mechanism of neurite inhibition by NG2, brevican and other CSPGs appear to differ. Interestingly, NG2 retains its ability to inhibit growth following chondroitinase ABC digestion, whereas brevican and some other, not-yet-identified CSPGs lose their inhibitory effects following digestion (Dou, Levine, 1994; Dow, Riopelle, 1994; McKeon et al., 1995; Snow et al., 1991; Yamada et al., 1997; Zuo et al., 1998). This suggests that the

mechanism of NG2's inhibitory influence may lie in the core protein whereas brevican's inhibitory influence depends upon the presence of its full length CS GAG chains. These differences in presentation, spatial and temporal expression and influences upon growth illustrate the diversity of the CSPG family. The wide diversity of the CSPG family may contribute to the conflicting evidence regarding CSPGs' influence upon axonal growth. As our understanding of proteoglycans expands, so does proteoglycan nomenclature.

Proteoglycan Nomenclature

Proteoglycans have been grouped in a variety of ways. One of the more traditional approaches is based upon their GAG constituents. CSPGs are an example of this type of proteoglycan nomenclature. Other groups of proteoglycans that are named based upon their GAG constituents are: keratan sulfate proteoglycans (KSPGs), heparan sulfate proteoglycans (HSPGs) and dermatan sulfate proteoglycans (DSPGs). By definition, proteoglycans that are in these groups each contain at least one of the following GAG chains: KS, HS or DS, respectively. However, a proteoglycan can have more than one type of GAG chain and thus, belong to more than one proteoglycan group.

Concurrent with an increase in core protein research, novel proteoglycan groups that are based upon core protein sequences rather than GAG constituents have been created. One example of a core protein-based proteoglycan group is the small leucine rich proteoglycans (SLRPs). As their name implies, the small core proteins of proteoglycans in this group are particularly rich in leucine (Ruoslahti, 1988). The members of this group include biglycan, decorin, lumican and fibromodulin (Kjellen, Lindahl, 1991b; Koops et al.,

1996). The first two proteoglycans listed also belong to the CSPG and DSPG group, whereas the last two proteoglycans belong to the KSPG group (Figure 1-1). Thus, a specific proteoglycan can belong to many different groups (Figure 1-1).

Proteoglycans are also grouped by core protein function. For example, the group of aggregating proteoglycans are characterized by a hyaluronic acid binding region (HABR) that enables these proteoglycans to attach to hyaluronic acid and form large macromolecule complexes (Margolis et al., 1996; Margolis, Margolis, 1994). Members of this family are: aggrecan, versican, brevican and neurocan (Margolis et al., 1996; Margolis, Margolis, 1994). Each of these aggregating proteoglycans belong to other proteoglycan groups including the CSPG family (Figure 1-1). This is another example of how members in various proteoglycan groups can overlap.

Members within any proteoglycan group can be different based upon their GAG constituents, core protein sequence, presentation to a neuron, and influence upon axonal growth. The great diversity in any of these groups can contribute to difficulties when examining the function of proteoglycans as a group rather than individually. For example, the majority of early CSPG studies used antibodies that bound to the CS GAG chains and could not distinguish distinct CSPGs (Brittis et al., 1992; Canning et al., 1996; Snow, Letourneau, 1992). Thus, it is likely that these CS GAG antibodies could be identifying a variety of CSPG members which may in turn, have separate, distinct influences upon neurite growth. Therefore, it is increasingly apparent that investigations of specific proteoglycans (defined by their core protein) are necessary. Some studies, including those

described in this dissertation, have begun to analyze distinct CSPGs and characterize their expression in the CNS.

The studies presented in Chapters 3 and 4 focus upon the specific CSPG, aggrecan. Aggrecan was selected for several reasons. This particular CSPG had not been identified in the CNS at the time that these studies began. Thus, the identification of aggrecan and many aggrecan degraditive fragments in the spinal cord (described in Chapter 3) was intriguing. In addition, several cell culture and developmental studies suggested that aggrecan could inhibit neurite outgrowth and cell migration (Peris et al., 1996; Pettway et al., 1996; Snow et al., 1996; Snow, Letourneau, 1992). Thus, aggrecan appeared to be a novel and promising CSPG to examine in the spinal cord.

Aggrecan

Aggrecan is a CSPG that has traditionally been associated with cartilage but is also present in lung (Juul et al., 1991), skin, cornea, aorta (Yamagata et al., 1993), brain (Li et al., 1996; Milev et al., 1998b) and spinal cord (Asher et al., 1995)(also described in Chapter 3). The core protein of aggrecan is large (210kD-350kD) and has three globular domains: G1, G2 and G3 (Figure 1-2)(Doege et al., 1991; Hascall et al., 1998; Neame, Sandy, 1994). The G1 domain is at the N-terminus of aggrecan and contains the HABR which qualifies this molecule as a member of the aggregating proteoglycans. Many aggrecan molecules can bind to HA and form large aggregates. The binding of aggrecan to HA is stabilized by two small link proteins (40kD) (Hardingham, Fosang, 1992) which bind to HA (Tengblad, 1981) and the G1 domain of aggrecan (Watanabe et al., 1997). In

addition to anchoring proteoglycans to HA, link proteins seem to play a role in the spacing of proteoglycans along the HA chain (Boyd et al., 1990; Perris et al., 1991) and appear to influence the size and shape of the aggregate (Blakemore et al., 1995; Kahn et al., 1994; Tang et al., 1996).

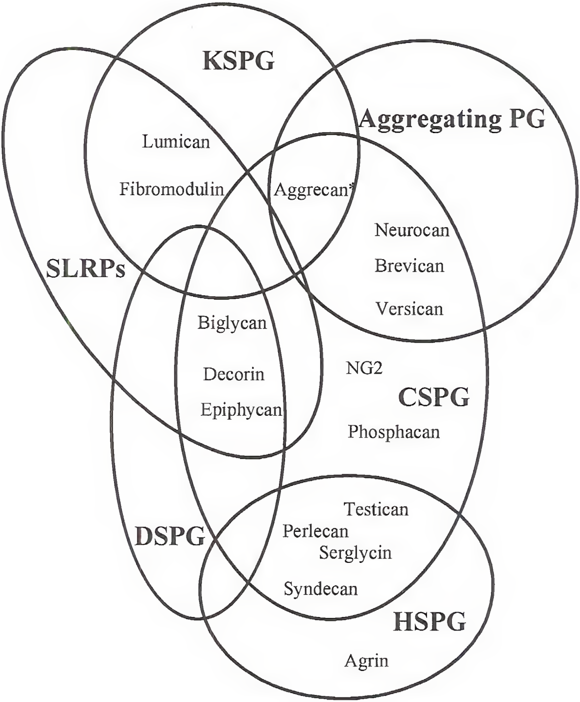
Although the G2 domain is highly homologous with the G1 domain, the G2 domain does not interact with HA and its function is presently unknown (Boyd et al., 1990; Fosang, Hardingham, 1989). The G3 domain is at the C-terminus of aggrecan and contains sequences that are similar to epidermal growth factor, hepatic lectin, and a complement regulatory protein. The function of the G3 domain is not well understood but this domain appears to be able to bind simple sugars (Roughley, Lee, 1994). The majority of the aggrecan core protein resides between the G2 and G3 domain. Over 100 GAG chains bind to this interglobular domain (Hascall et al., 1998). The GAG chains extend away from the protein core like bristles of a brush. The high anionic charge of the GAG chains enable the molecule to bind a large quantity of water. These properties of aggrecan enable this molecule to withstand compressive loads (Sandy et al., 1996) and is believed to contribute to the turgor and weight-bearing abilities of cartilage. In the CNS, aggrecan may serve to maintain the integrity of CNS tissue and may buffer against pressure (Hildebrand et al., 1994). In addition, this large molecule could sterically hinder or block neurite growth. Currently, the role(s) of aggrecan in the CNS is (are) not clear but studies have begun, including the ones described in this dissertation, that focus on the effects of aggrecan upon axonal growth.

Earlier studies have suggested that aggrecan or an aggrecan-like protein may exist in the CNS (Asher et al., 1995; Bignami et al., 1993; Domowicz et al., 1995; Fryer et al., 1992; Li et al., 1996; Milev et al., 1998b; Pettway et al., 1996). A subset of these indicate that aggrecan or an aggrecan-like protein may serve as a guidance molecule during development (Oohira et al., 1994; Perris et al., 1996; Pettway et al., 1996). For example, neural crest cells avoid the aggrecan-like perinotochord region in the chick (Oettinger et al., 1985; Yamagata et al., 1993). Soluble aggrecan also decreases neural crest cell migration from the neural tube *in vitro* (Perris et al., 1996). This inhibitory effect is diminished by preincubation of aggrecan with HA fragments or antibodies against the G1 domain of aggrecan that block the HABR (Perris et al., 1996). It also seems that aggrecan may inhibit neurite outgrowth *in vitro*. Previous studies that had demonstrated that CSPGs could inhibit neurite outgrowth *in vitro* have now identified the CSPG used in the study as aggrecan (Snow et al., 1996; Snow, Letourneau, 1992; personal communication with Diane Snow).

The experiments described in this dissertation are grouped into three sections: 1) evaluation of the general expression of CSPGs, 2) analysis of aggrecan core protein in the spinal cord and 3) evaluation of aggrecan's influence upon axonal growth *in vivo*. Chapter 2 (Lemons et al., 1999) describes our initial studies that characterized general expression in the normal and contused spinal cords as well as following intraspinal transplantation of embryonic spinal cord tissue. These studies were important in establishing a correlational relationship between increases in CSPG expression with limited axonal growth. These studies led to the experiments described in Chapter 3 and 4 that

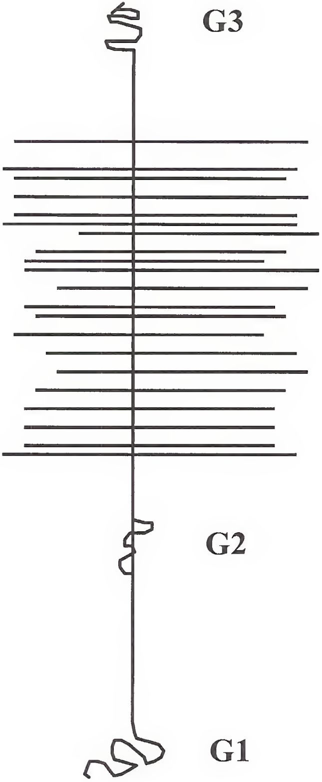
focus upon the expression and function of aggrecan, a member of the CSPG family. Our data indicates that aggrecan is present in the normal and injured spinal cord and inhibits axonal growth *in vivo*. Thus, aggrecan may be one of the inhibitory environmental factors that contributes to the lack of regeneration following spinal cord injury. Our studies also suggest that the inhibitory effects of aggrecan are not significantly diminished following chondroitinase ABC digestion. Thus, the ability of this enzyme to cleave CSPGs (Lemons et al., 1999) (also described in Chapter 2) may not be an effective method to diminish aggrecan's growth inhibitory effect *in vivo*. The documentation of aggrecan in the spinal cord, in combination with its growth inhibitory influences *in vivo*, qualify this molecule as one of the possible impediments to regeneration in the injured CNS.

Figure 1-1. Euler circle diagram illustrates the overlap of members of several proteoglycan groups.



* Some sources of aggrecan (e.g. bovine cartilage) contain KS and CS GAG chains. Purified aggrecan (aA1D1) from RCS only has CS GAG chains.

Figure 1-2. Diagram of aggrecan molecule. The protein core (represented by a vertical line) has three globular domains: G1, G2 and G3. The area between G2 and G3 can have approximately 100 CS GAG chains (represented by horizontal lines). Aggrecan can also have some KS GAG chains and *N* and *O* linked oligosaccharides attached to its protein core (not shown).


G3
G2
G1

CHAPTER 2
CHONDROITIN SULFATE PROTEOGLYCAN IMMUNOREACTIVITY INCREASES FOLLOWING SPINAL CORD INJURY AND TRANSPLANTATION

Introduction

Failure of the adult central nervous system (CNS) to regenerate following injury is poorly understood. Although mature, injured CNS neurons have an intrinsic capacity to regenerate, this capacity appears to be restricted, at least in part, by the extracellular environment (Benfey, Aguayo, 1982; Richardson et al., 1984; So, Aguayo, 1985). Several putative growth inhibitory molecules have been identified (Bachmann et al., 1995; Caroni, Schwab, 1988a; Caroni, Schwab, 1988b; Pesheva et al., 1994; Schnell, Schwab, 1990; Schwab, Schnell, 1991; Steindler et al., 1995; Taylor et al., 1993), including the family of chondroitin sulfate proteoglycans (CSPGs). The inhibitory nature of CSPGs is suggested by several developmental studies (Fichard et al., 1991; Landolt et al., 1995; Oakley et al., 1994; Oakley, Tosney, 1991; Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990), *in vitro* assays (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Dou, Levine, 1995; Perris, Johansson, 1987; Rudge, Silver, 1990; Snow, Letourneau, 1992; Yamada et al., 1997) and in a few correlative studies in the adult (Davies et al., 1997; Fitch et al., 1996; Gates et al., 1996).

In the developing brain and spinal cord, migrating cells and growing axons avoid CSPG expressing areas (Oakley et al., 1994; Oakley, Tosney, 1991; Pettway et al., 1996; Pettway et al., 1990). It appears that these CSPG positive areas form boundaries that help

guide or channel migrating cells and growing axons to their appropriate destinations. Once targets are reached and innervated, the end of further growth is correlated with an increase in CSPG-IR (Dow et al., 1994) around the connected axons (Pindzola et al., 1993; Steindler, 1993). Thus, CSPGs may be important in repulsing axonal growth away from an inappropriate target and/or preventing axons from growing to other areas once they have reached their destination.

An inhibitory role for CSPGs is also supported by *in vitro* studies in which a variety of neuronal populations prefer to attach, migrate and extend processes on non-CSPG substrates versus CSPG-positive substrates (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Katoh-Semba et al., 1995; Oohira et al., 1991; Perris, Johansson, 1987; Snow, Letourneau, 1992). The ability of CSPGs to inhibit growth can be affected by a variety of factors including the source and age of the neurons studied, the presentation and type of CSPGs, and the relative ratio of CSPGs to other molecules (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992). These differences may account for the discrepancies in earlier *in vitro* studies which report that CSPGs do not inhibit growth (Dow et al., 1993; Faissner et al., 1994; Iijima et al., 1991).

In vitro, the growth inhibitory effects of CSPGs can be diminished by enzymatic digestion with chondroitinase ABC (Bovolenta et al., 1993; Brittis et al., 1992; Canning et al., 1996; McKeon et al., 1995; McKeon et al., 1991; Perris et al., 1996). Chondroitinase ABC specifically digests the chondroitin sulfated glycosaminoglycan side chains on the protein core of the CSPG molecule. This enzymatic digestion results in greater neurite growth of fetal dorsal root ganglion cells on chondroitinase ABC-treated spinal cord or brain extracts than on non-treated extracts (Dow et al., 1994; McKeon et al., 1995).

CSPGs are present in the adult CNS (Asher et al., 1995; Bignami et al., 1992; Margolis, Margolis, 1993; Ruoslahti, 1988). Following injury, increases in the expression of CSPGs have been reported in the adult cerebral cortex (Fitch et al., 1996; McKeon et al., 1991), cerebellum (Levine, 1994), dorsal root entry zone (Pindzola et al., 1993), and spinal cord (Fitch et al., 1996) using immunocytochemistry techniques. These injury-induced increases in CSPG expression were correlated with a lack of growth *in vivo* (Davies et al., 1997; Fitch et al., 1996; Gates et al., 1996). The ability of CSPGs to inhibit growth is further suggested by cell culture explant studies. For example, neurite outgrowth of embryonic retinal neurons plated *in vitro* on injured adult brain explants containing high CSPG levels was significantly less than the neurite outgrowth on injured neonatal brain explants, with lower CSPG levels (McKeon et al., 1991). Furthermore, neurite outgrowth on CSPG-positive injured adult brain explants was increased following chondroitinase ABC digestion (McKeon et al., 1995). Correlational data from *in vivo* studies, combined with results from cell culture studies, suggest that the injury-induced levels of CSPGs may contribute to the limited growth that occurs following injury in the adult CNS.

Relatively little is known about the expression of CSPGs following spinal cord injury or intraspinal transplantation. Therefore, the aims of this study were to: (1) study the spatial and temporal expression profile of CSPGs following spinal cord contusion injury in the adult rat; (2) determine if chondroitinase ABC can be used to degrade CSPGs *in vivo;* and (3) study the spatial and temporal expression profile of CSPGs following intraspinal transplantation of fetal spinal cord tissue into subchronic spinal cord contusion cavities. Our results indicate that marked increases in the immunoexpression of CSPGs occur

rapidly following injury, persist for extended periods and that CSPGs can be cleaved *in vivo* by exogenous application of chondroitinase ABC. In addition, neither the rapid increase or the long-term elevation of CSPGs in the adult spinal cord appear to be altered by the presence of an intraspinal transplant of fetal spinal cord tissue. Furthermore, the fetal graft, which initially has relatively low levels of CSPG-IR, develops robust CSPG labeling comparable to that seen in the injured host.

Methods

Seventy-seven adult Long Evans rats were used in these studies for three sets of experiments. In the first set, twenty-three rats received low thoracic spinal cord contusion injuries using the New York University (NYU) impactor device (Gruner, 1992). These animals, in groups of at least four, were sacrificed at seven, ten, thirteen, thirty or forty days post-injury. Nine additional animals that received either laminectomies-only (n=6) or no surgical procedure (n=3), served as controls. The laminectomies-only rats were sacrificed at seven and thirty days post-surgery.

In the second set of experiments, twenty rats received low thoracic contusion injuries followed by either exogenous applications of chondroitinase ABC (n=11) or saline (n=9) during the same surgical procedure. An additional group of eighteen rats, without contusion injuries, had chondroitinase ABC (n=9) or saline (n=9) applied to their intact spinal cords. At least three animals in each of the four groups (contusion plus chondroitinase or saline and non-contusion plus chondroitinase or saline) were sacrificed at four, six and eight days post-surgery.

In the third set of experiments, seven rats received intraspinal transplants of a fetal spinal cord tissue suspension in addition to spinal cord contusion injuries. The transplants were placed ten days post-contusion injury. Three of these animals were sacrificed at three days post-transplantation (thirteen days post-contusion injury) and four were sacrificed at thirty days post-transplantation (forty days post-contusion injury).

The low thoracic spinal cords of all rats in all experiments were examined using general cell stains and immunocytochemistry. In immunocytochemistry procedures, a monoclonal antibody against chondroitin-6-sulfate proteoglycan (International Chemical and Nuclear, ICN) and a polyclonal antibody against GFAP (Accurate) were used.

Spinal Cord Contusions

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (35 mg/kg for females and 40 mg/kg for males). Under aseptic conditions, a low thoracic laminectomy exposed the spinal cord. The spinal segment was contused by dropping a ten gram rod twenty-five mm onto the exposed spinal cord using the NYU impactor device (Gruner, 1992). Following contusion, the injury site was covered with durafilm to mark the lesion area and to minimize connective tissue adhesions to the dura. Muscle was closed using absorbable sutures. The skin was closed using stainless steel surgical staples. Post-operatively, animals received three cc saline subcutaneously and recovered on a heating pad. Rats also received 50 mg/kg of an antibiotic, keflin or penicillin G, on the day of surgery and once a day for seven to ten days following surgery. Rats' bladders were manually expressed twice a day following spinal cord injury until bladder function returned or until sacrifice.

Chondroitinase ABC Application

Chondroitinase ABC-absorbed gelfoam was applied to the dorsal aspects of twenty spinal cords. Eleven of these spinal cords received a contusion injury immediately prior to gelfoam application and nine did not receive a contusion-injury prior to gelfoam application. The dorsal aspect of the spinal cord was exposed by a laminectomy procedure. Those animals that received a contusion injury were placed into the NYU impactor device and sustained a dynamic weight drop onto the exposed spinal cord as previously described. Following injury, the dura mater was slit longitudinally and the gelfoam was placed on the dorsal aspect of the spinal cord. The dura matter of those animals that did not receive a contusion injury was slit in the same manner immediately after a laminectomy procedure. When the edges of the dura mater were still visible and intact, the dura mater was sutured with 8-0 silk to hold the gelfoam in place. Durafilm was placed over the sutured dura. If the dura mater could not be sutured, durafilm was placed over the gelfoam and its edges were tucked under the remaining dura matter. As a control, eighteen animals received saline absorbed gelfoam in the same procedure as described for chondroitinase ABC absorbed gelfoam. Nine of these animals received a contusion injury, nine did not. At least three animals in each saline control group were sacrificed at four, six and eight days post-surgery. All animals receiving chondroitinase ABC or saline absorbed gelfoam were evaluated for *in vivo* CSPG cleavage using immunocytochemical procedures.

Preparation of Fetal Spinal Cord Grafts

Time-pregnant Long Evans rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50mg/kg) fourteen days after conception (E14). The intact uterus was removed by a ovariohysterectomy procedure and placed on ice. Under sterile conditions on ice, the fetuses, and then their spinal cords, were removed using a dissecting microscope. The dura mater and spinal ganglia were stripped from the spinal cord and the spinal cords were then minced in Dulbecco's modified Eagle medium (DMEM from Gibco). The minced pieces were triturated with a pipette into a thick suspension. The suspension was kept on ice until used for transplantation.

Fetal Transplantation

Intraspinal grafts were performed ten days following spinal cord contusion. Animals were anesthetized as described earlier and the site of injury re-exposed. Using a Hamilton syringe with a 30 gauge needle, 20-30 μls of an E14 spinal cord suspension were slowly injected into the center of the injury site until reflux occurred. Following transplantation, durafilm was placed over the graft injection site. The muscle and skin were closed in layers.

Perfusions

Rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with 0.9% saline and 4% paraformaldehyde (pH, 7.4) in 0.1 M phosphate buffer. The spinal cords were removed and placed in 30% sucrose in phosphate buffer (pH, 7.4). Tissue was frozen and sectioned on a cryostat at 14μm. Selected sections were stained with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R,

Fluka, New York) stains. The remaining sections were processed for immunocytochemistry.

Immunocytochemistry

Chondroitin-6-sulfate proteoglycan

Spinal cord tissue was washed with Tris-buffered saline (pH, 7.2) and then incubated with 0.5 U chondroitinase ABC (Sigma)/ 1 ml of 0.5 M NaCl Tris buffer (pH, 8) for three hours at 38°C. Sections were rinsed with 1% horse serum in Tris-buffered saline containing 0.4% Triton X-100 (Sigma) (1%HS-TBS-T) with the pH adjusted to 7.2. Following a 10% horse serum block for one hour, the tissue was incubated with a monoclonal chondroitin-6-sulfated proteoglycan (C6SPG) primary antibody (#63-652-1 from ICN, 1:1,000 in 1%HS-TBS-T) overnight at 4° C. This monoclonal antibody specifically recognizes the remaining sugar stub on the protein core of chondroitin-6-sulfate proteoglycan molecules that are exposed following enzymatic digestion by chondroitinase ABC. The next day the tissue was thoroughly rinsed with 1%HS-TBS-T and incubated with a biotinylated horse antimouse antibody conjugated to FITC (FI2001 from Vector Laboratories, 1:100) for one hour. The tissue was again thoroughly rinsed with 1%HS-TBS-T and fixed with 4% paraformaldehyde (pH, 7.4) for ten minutes to ensure stable binding. Tissue that was labeled for CSPG-only was subsequently rinsed in 0.1 M PB (pH, 7.4) and cover slipped in flourescent mounting media (S3023, Dako). All tissue specimens were viewed with a Zeiss Axiophot microscope equipped with fluorescent optics. The IR patterns described from these experiments are referred to as CSPG-IR. Due to the distinct labeling pattern of CSPGs in the dorsal root where it

surrounds axons (see current results), CSPG-IR was examined in the dorsal roots that remained attached to the spinal cord tissue and served as an internal positive control.

Glial fibrillary acidic protein

In some animals, tissue sections adjacent to those labeled for CSPG were processed using an antibody against GFAP. This tissue was rinsed in 0.1 M PB and subsequently washed in 1% goat serum in phosphate-buffered saline containing 0.4% triton X-100 with a pH, 7.2 (1%GS-PBS-T). Following a 10% goat serum block for one hour, the tissue was incubated with the polyclonal GFAP antibody (AXL 457 from Accurate, 1:2,000 in 1%GS-PBS-T) overnight at 4° C. On the second day, the tissue was washed with 1%GS-PBS-T, incubated with a biotinylated goat antirabbit antibody conjugated to Texas Red (TR1000 from Vector Laboratories, 1:100) for one hour and washed again with 1% GS-PBS-T. The sections were rinsed with 0.1 M phosphate buffer and cover slipped with flourescent mounting medium. All tissue specimens were viewed with a Zeiss Axiophot microscope equipped with fluorescent optics.

***In Vivo* CSPG cleavage**

In order to detect *in vivo* CSPG cleavage, CSPG immunocytochemical procedures were slightly modified. For these studies, chondroitinase ABC was not applied to the fixed spinal cord tissue sections and therefore, CSPGs were not cleaved during immunocytochemistry procedures. This is a critical point because the CSPG antibody used in these studies only binds to chondroitinase ABC digested chondroitin-6-sulfate proteoglycans and does not bind to intact CSPG molecules. Therefore, when this CSPG antibody was applied to spinal cord tissue that was not treated with chondroitinase ABC during immunocytochemistry procedures, this antibody can only bind to those chondroitin-

6-sulfate proteoglycan molecules that were previously cleaved *in vivo* by exogenously applied chondroitinase ABC. In this situation, positive staining selectively identifies those CSPG molecules that were cleaved *in vivo* by chondroitinase ABC. As a negative control, chondroitinase ABC was not applied during immunocytochemistry procedures on tissue from animals that did not receive an application of exogenous chondroitinase ABC *in vivo*. When chondroitinase ABC is not applied *in vivo* or during immunocytochemical procedures, the CSPG antibody used in this study cannot bind to its antigen.

Results

CSPG-IR Increases Following Spinal Cord Injury

In order to determine if CSPGs are present in a temporal and spatial pattern that is consistent with a growth-inhibitory role in the injured spinal cord, immunoreactivity for chondroitin-6-sulfate proteoglycan was evaluated in the spinal cords of three groups of animals: 1) normal, 2) laminectomy-only and 3) spinally contused. Immunocytochemical data show that CSPGs are present in both the spinal gray and white matter of normal animals (Figure 1A). CSPG-IR is seen throughout the gray matter with the exception of the intracellular spaces of various sized neurons. This is particularly apparent in the ventral horn (Figure 1A). In the white matter, CSPG staining appears to discretely outline and fill the spaces between the axons creating a honeycomb-like staining pattern (Figure 1C). CSPG-IR is also present along the edge of the normal spinal cord where astrocytic endfeet form a CSPG-containing-basement membrane (not shown). CSPG positive staining is also seen in the basal lamina that lines blood vessels (not shown). CSPG-IR in

the spinal cords of controls that underwent a laminectomy-only procedure were non-distinguishable from normal, non-operated controls.

At seven days post contusion-injury, CSPG-IR is greatly intensified (Figure 1B) compared to normal and laminectomy-only controls. In the white matter, damaged and swollen axonal profiles in the white matter are heavily outlined and surrounded by CSPGs (Figure 1D). In contrast to the white matter, it is difficult to determine if the level of CSPGs in the gray matter increases because the normal baseline of CSPGs in the gray matter is high. It is evident, however, that following injury, the level of CSPGs does not decrease in the gray matter. If CSPGs do increase in the gray matter, it is most likely to occur immediately adjacent to the lesion cavity. Any spared host tissue at the cavity edge, whether it is gray or white matter, is intensely labeled for CSPGs. CSPG-IR at ten, thirteen, thirty and forty days post-injury is very similar to that seen at seven days post-injury with one exception. At thirty and forty days post-contusion, the intensity of CSPG-IR in the ventral half to one-third of the white matter appears less intense compared to the persistent, robust CSPG-IR in the dorsal half to two-thirds of the spinal cord and around the entire lesion edge (in both the ventral and dorsal halves) (Figures IE, F). CSPG-IR, therefore, increases within the first week following spinal cord contusion injury and remains elevated, at least in part, for at least forty days.

Cleaved CSPG Molecules are Detected *In Vivo* Following Chondroitinase ABC Application

In order to determine if CSPGs could be broken down in a living animal, gelfoam absorbed with the bacterial enzyme, chondroitinase ABC, was placed and left on the dorsal aspects of rat spinal cords for four to eight days. To detect CSPG molecules that

Figure 2-1. CSPG-IR in normal and injured adult rat spinal cord tissue cross sections. CSPG-IR in the left ventral quadrant of a normal spinal cord (A). CSPG-IR is seen throughout the gray matter with the exception of the intracellular space of some small and large neurons. CSPG-IR is also seen at low levels in the white matter. At a higher magnification in the white matter, it appears as though CSPG-IR surrounds axonal profiles (C). Following injury, increases in CSPG-IR are most apparent in the white matter of the injured spinal cord segment. A comparison of the left ventral quadrant of a spinal cord seven days post-contusion (B) with a normal ventral quadrant (A) shows the striking increase. Higher magnifications of normal ventral white matter (C) and ventral white matter seven days post-injury (D) further illustrate the dramatic difference in CSPG-IR pre- and post-op. By thirty days post-contusion injury (E), CSPG expression in the ventral two-thirds of the spinal cord appears less intense compared to the CSPG-IR pattern at seven days post-injury (B). CSPG-IR, however, remains intense along the cavity edge and in the dorsal and lateral aspects of the remaining white matter (E). A higher magnification, 30 days post-contusion, shows the intensity of CSPG-IR, in both white and gray matter, at the lesion edge (F). Figures 1E and 1F are montages. WM or ** = white matter; VH or * = ventral horn.

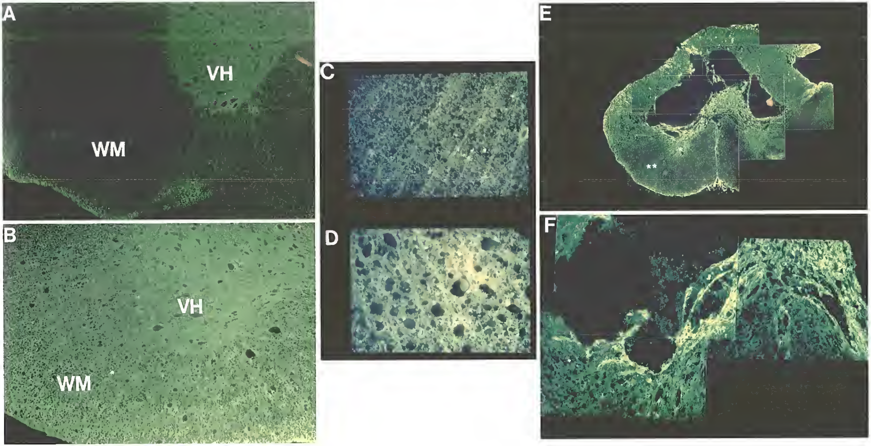
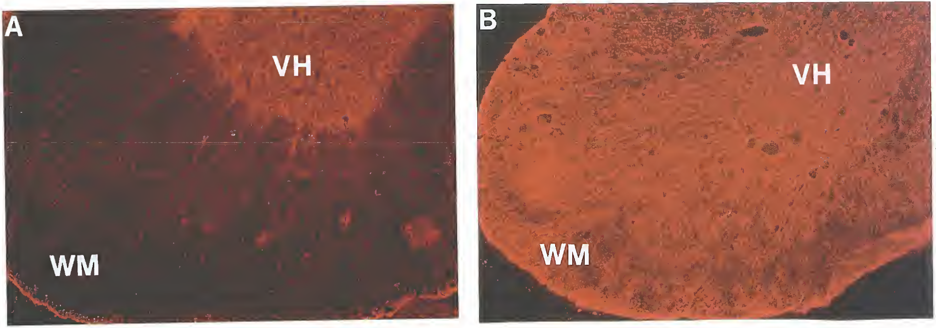

A
VH
WM
B
VH
WM
C
D
E
**
F

Figure 2-2. GFAP-IR in ventral quadrant of normal (A) and contused (B) spinal cord of adult. GFAP-IR is intense in contused spinal cord at seven days post-injury. VH=ventral horn, WM=white matter


A
VH
WM
B
VH
WM

had been cleaved *in vivo* by chondroitinase ABC absorbed gelfoam treatment, chondroitinase ABC was **not** applied during immunocytochemistry procedures performed on the fixed spinal cord sections. The CSPG antibody used in these studies specifically binds to a site on CSPGs that is exposed by chondroitinase ABC digestion and can not bind to intact CSPG molecules (see methods). Therefore, when chondroitinase ABC was not applied to fixed spinal cord sections, this antibody could only bind to those CSPG molecules that were cleaved as a result of exogenous chondroitinase ABC treatment *in vivo*. No positive immunoreactivity was seen when normal and injured-only tissue was not incubated with chondroitinase ABC. Thus, this approach can be used to selectively identify CSPG molecules that were cleaved *in vivo*. Any immunoreactivity seen without chondroitinase ABC application during immunocytochemistry procedures is referred to as "CSPG cleavage". In contrast, the CSPG-IR patterns described thus far in the normal and injured animals are a result of chondroitinase ABC application to fixed spinal cord tissue sections during the immunocytochemistry procedures. In this way, the CSPG antibody can label CSPG molecules present within the spinal cord tissue. Immunoreactivity patterns seen following chondroitinase ABC application during immunocytochemistry procedures will continue to be referred to as "CSPG-IR".

In vivo CSPG cleavage is seen at four and six days, but not at eight days, post-surgery in the spinal cords of all rats that received chondroitinase ABC gelfoam treatment. At four days, cleaved CSPGs are present in the dorsal rootlets, dorsal columns, the superficial dorsal horns and superficial lateral and ventral white matter (Figure 2-3B, C). The extent of this circumferential rim of cleavage varied from animal to animal. However, there was never any cleavage seen in the center of the spinal cord. At six days post-

surgery, *in vivo* CSPG cleavage is less robust and restricted to the superficial dorsal aspect of the spinal cord in all chondroitinase ABC treated animals. At eight days following surgery, *in vivo* CSPG cleavage is not observed in any of the animals.

As a control, saline absorbed gelfoam was applied to the dorsal aspect of spinal cords. Half of the saline-treated animals received contusion injuries prior to saline application and the remaining half did not. *In vivo* CSPG cleavage was not detected at four, six or eight days post-surgery in the spinal cords of any of the saline-treated animals (Figure 2-3A). In addition, CSPG cleavage was not detected in the spinal cords of any animal that did not receive chondroitinase ABC-treatment such as normal, non-operated animals and contusion-only animals.

In addition to assessing CSPG cleavage, CSPG-IR was also examined in all animals that received chondroitinase ABC or saline absorbed gelfoam application. This was initially done to test if chondroitinase ABC affected overall CSPG immunoexpression. Our results show that CSPG-IR is not altered by chondroitinase ABC. Our results do demonstrate, however, that CSPG-IR is dramatically affected by the gelfoam treatment. Interestingly, CSPG-IR was increased in all animals that received either saline or chondroitinase ABC gelfoam treatment compared to normal, un-operated controls and laminectomy-only controls (with no gelfoam treatment). Although an increase was expected in the contusion injured spinal cords, it was unanticipated in the animals that did not receive a contusion injury. Cresyl violet stained sections of the non-contused spinal cords reveal a mild tissue disruption in spinal cord segment that was under the gelfoam. This tissue damage and increase in CSPG-IR were not seen in sham operated (laminectomy-only) rats (not shown).

This suggests that the gelfoam placed on the spinal cord and secured under the dura may cause a compression-type injury that results in an increase in CSPG-IR.

CSPG-IR Following Intraspinal Transplantation

CSPG-IR was evaluated in the spinal cords of eight adult rats that had fetal spinal cord grafts placed into their lesion cavities at ten days post-injury. This was done to determine: 1) if the graft altered CSPG-IR in the host spinal cord and 2) if the CSPG-IR pattern in the transplanted spinal cord suggested that CSPGs could be contributing to the limited growth between graft and host (Jakeman and Reier, 1991). Three days following transplantation (thirteen days post contusion injury), the host spinal cord shows elevated levels of CSPG-IR. In the four animals examined at this time point, the pattern of CSPG-IR in the host does not appear to be different from that seen at thirteen days following injury. CSPG-IR is intense throughout the white matter and along the lesion edge that consists of white and gray matter (Figure 2-4 C) compared to normal and laminectomy-only controls. Although the entire neuropil is robustly CSPG immunopositive, some CSPG-IR around the lesion edge is attributable to blood vessels. Blood vessels are CSPG-IR because they are lined with CSPG containing basal lamina.

Prior to placement into a contusion lesion, an E14 spinal cord has low levels of CSPG-IR (Figure 2-4 B). The most robust CSPG positive staining in an E14 spinal cord is localized to the vessel-rich arachnoid and basal lamina that surround the spinal cord; however, some CSPG-IR is detectable in the medial/ventral aspects of the embryonic spinal cord. By three days post-transplantation, the fetal spinal cord graft has begun to develop some CSPG-IR. Overall, however, CSPG-IR is low or barely detectable. In some areas of the transplant, CSPG-IR can be seen in the walls of blood vessels and in a few areas in the

Figure 2-3. Detection of *in vivo* CSPG cleavage in animals that received chondroitinase ABC application. A high power magnification of the dorsal columns in a cross section of spinal cord from a saline control shows an absence of cleaved CSPGs (A). In contrast, cleaved CSPGs are seen in a similar view of the dorsal columns four days post-chondroitinase ABC application (B). A montage shows that cleaved CSPGs are also present in the lateral and ventral rim of the spinal cord, at four days post-chondroitinase ABC application (C).

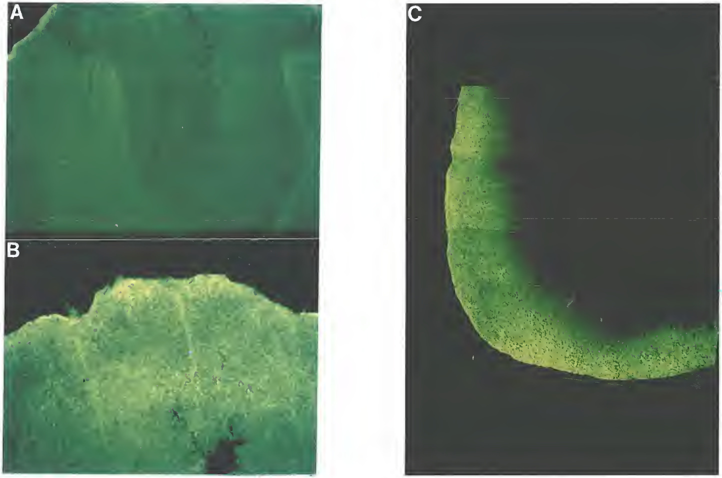

A
B
C

Figure 2-4. CSPG-IR in contused spinal cords that received an E14 spinal cord transplant. A cresyl-violet stained cross section through the transplant shows a well integrated, neuron-abundant, highly vascularized, embryonic graft filling the contusion cavity at thirty days post-transplantation (A). The E14 spinal cord tissue used for transplantation shows no CSPG-IR (B). CSPG-IR is seen in the basal lamina that outlines the developing spinal cord and in areas adjacent to the developing nervous system. These CSPG-IR structures are stripped away prior to transplantation. At three days post-transplantation, very low CSPG levels are detectable in the graft (C). The low CSPG-IR in the graft is in stark contrast to the host tissue that is intensely labeled for CSPGs (C). The intense CSPG-IR patterns in the host are similar to those seen in time matched injury controls. At thirty days following transplantation, robust CSPG-IR has developed throughout the fetal graft (D). The arrows indicate the host-graft border; WM = white matter; T= transplant; vh = ventral horn; dr = dorsal root; dg = dorsal root ganglia.

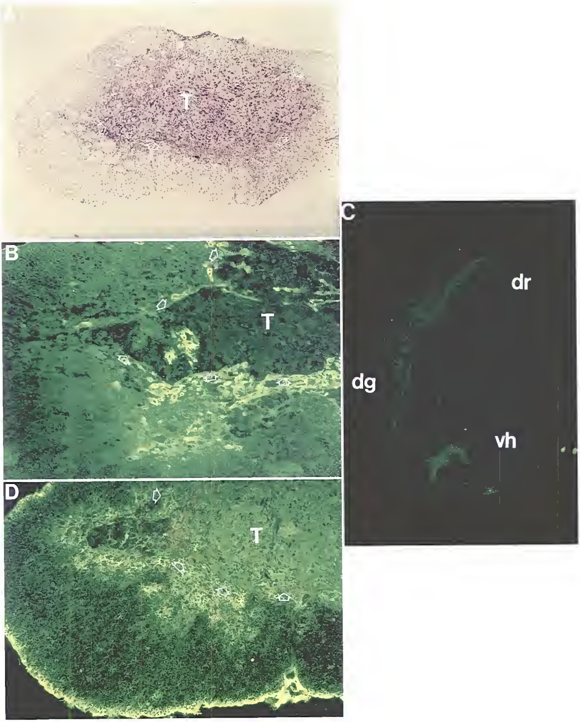
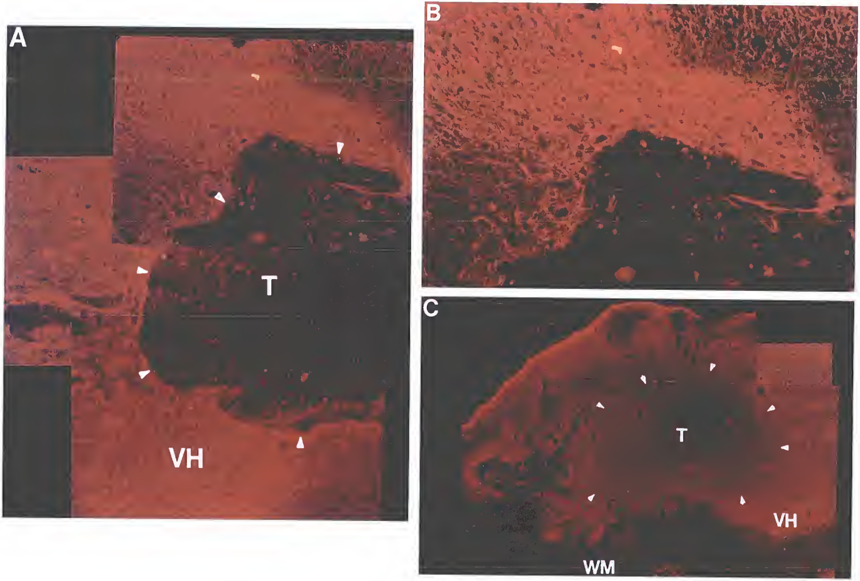

T
B
T
C
dr
dg
vh
D
T

Figure 2-5. GFAP-IR following intraspinal placement of E14 spinal cord tissue into contusion injured rats. At three days post-transplantation, GFAP-IR is intense throughout the host white and gray matter (A). However, GFAP-IR is barely detected within the embryonic graft (A, B). These trends are also seen in one of the photomircographs (B) used to make the montage (A). At thirty days post-transplantation, GFAP-IR continues to be intense in the dorsal aspect of the host spinal cord but has decrease in the ventral host white matter (C). GFAP-IR is present in most areas of the graft. Both figures are montages. Arrows indicate host-graft border; WM = white matter; VH = ventral horn; T = transplant.


A
T
VH
B
C
T
VH
WM

neuropil that have detectable, but low levels of CSPG-IR (Figure 2-4 C). The non-vessel areas in the graft that are CSPG-positive at this time are few and are considerably lower in intensity than the injured host spinal cord.

By thirty days post-transplantation (forty days post-contusion injury), the fetal graft is highly vascularized, contains many neurons and is well integrated with the host (Figure 2-4A). The graft is completely and intensely CSPG positive and CSPG-IR in the host continues to be intense in the same pattern as CSPG-IR in the time-matched contusion-only animals (Figure2-4D). Thus, although the CSPG-IR in the transplant does appear to change over time, it does not appear to alter the intensity of CSPG-IR in the host at three or thirty days post-transplantation. The fairly rapid increase in CSPGs in both the host and graft may create an environment that deters growth. This could account for the limited connectivity that is seen with intraspinal transplantation in the adult.

Similar Distributions of GFAP-IR and CSPG-IR

Astrocytes have been implicated in the production of CSPGs (Asher et al., 1998; Geisert et al., 1998; Johnson et al., 1992; Koops et al., 1996; McKeon, Nopachi, 1998). In order to determine if astrocytes are present in areas of CSPG production, some sections adjacent to those labeled for CSPGs were processed for GFAP-IR. GFAP-IR patterns are very similar to those seen for CSPG following both spinal cord contusion injury and intraspinal transplantation. At seven days post contusion-injury, GFAP-IR is intensified compared to normal and laminectomy-only controls (Figure 2-2A, B). GFAP-IR at later time points is very similar to that seen at seven days post-injury with one exception. At thirty and forty days post-contusion, GFAP-IR in the ventral half to one-third of the white matter appears similar to that seen in controls. This is in contrast to the more central and

dorsal areas that remain intensely immunoreactive. The same early global increases in GFAP-IR are seen at three days following intraspinal transplantation (Figure 2-5 A, B). This time point is equivalent to thirteen days post-contusion injury. By thirty days post-transplantation (forty days post-contusion injury), a decrease, that parallels that seen following injury-only, is apparent in the ventral white matter areas (Figure 2-5 C). The transplanted tissue also shows GFAP-IR. At three days post-transplantation, GFAP-IR, like CSPG-IR, is relatively low in the graft (Figure 2-5A). By thirty days post-transplantation, GFAP-IR has increased in the graft (Figure 2-5C). This increase is not uniform throughout the graft nor as robust as in the host. Thus, GFAP-IR increases within the first week following spinal cord contusion injury, remains elevated, at least in part, for forty days and has a spatial distribution in the host that is similar to CSPGs'. Intraspinal grafts of E14 spinal cord tissue do not alter GFAP-IR in the host. GFAP-IR in the grafts, themselves, also increases following transplantation.

Summary of Results

In these studies, we have shown that CSPG-IR is dramatically increased following spinal cord contusion injury. This increase occurs by four days post-injury (earliest time point evaluated) and persists around the lesion edge and in the dorsal half to two-thirds of the spinal cord for at least forty days (the longest time point evaluated). Similar spatial distributions are also seen for GFAP-IR. Using chondroitinase ABC, the injury-induced CSPGs can be cleaved *in vivo*. Cleaved CSPG molecules are detected at four and six days post-chondroitinase ABC treatment but are not detected in any saline-treated animals. In addition, CSPG-IR remains elevated in the host following intraspinal transplantation of E14 spinal cord into semi-chronic contusion cavities. Elevations in GFAP-IR are apparent in

the same areas of the host as CSPG. CSPG-IR is low compared to the intense labeling in the surrounding tissue at the time of transplantation. Low levels of CSPG-IR are seen in a few areas of the graft at three days post-transplantation compared to the intense CSPG-IR in the host. By thirty days post-transplantation, CSPG-IR is intense and robust throughout the entire graft. The presence of CSPG-IR is accompanied by GFAP-IR at both early and late time points.

Discussion

Putative Inhibitory Molecules Increase and Persist Following Spinal Cord Injury

A marked increase in CSPG-IR is present at four days following spinal cord contusion and persists for at least forty days. These results with a contusion-injury model are consistent with other studies that report an increase in CSPG-IR following different types of injury in both the brain and spinal cord (Davies et al., 1997; Fitch et al., 1996; Levine, 1994; McKeon et al., 1991; Pindzola et al., 1993). The results in this paper extend these findings by showing an increase in CSPG-IR several days earlier and at least a month longer than has been reported previously for spinal cord injury. The most persistent CSPG-IR increases were seen in the dorsal two thirds of the contused spinal cord. At thirty and forty days post-injury, when CSPG-IR in the dorsal two thirds was as intense as at four days post-injury, CSPG-IR in some ventral white matter areas had decreased to near-normal levels. It is likely that the spatial differences in the long term expression of CSPGs are a result of the injury method. The impactor device used in these studies drops a weight directly onto the exposed dorsal aspect of the spinal cord. Although this affects the entire spinal cord, as indicated by the initial global increases in CSPG-IR throughout both ventral

and dorsal areas, the injury is more damaging to the dorsal spinal cord. We (Figure 2-1E), as well as others (Basso et al., 1996; Beattie et al., 1997), show that the cysts which occur as a result of a 25mm injury with the NYU impactor device typically are located more dorsally with the greatest tissue preservation seen ventrally. Thus, the greater dorsal damage is correlated with a more persistent increase in CSPGs.

Increased CSPG expression could stem from a variety of cellular events that occur as a consequence of spinal cord injury. These cellular events include inflammatory reactions, as well as scar and cavity formation (Anderson, Hall, 1994; Basso et al., 1996; Carlson et al., 1998; Popovich et al., 1997; Streit et al., 1998). Among the inflammatory events, transcripts of several cytokines increase following spinal cord contusion injury (Streit et al., 1998). Increases in cytokine levels may contribute to increased CSPG expression. Many cytokines can stimulate astrocytes to upregulate CSPG production *in vitro* (Asher et al., 1998; Geisert et al., 1998; Johnson et al., 1992; Koops et al., 1996; McKeon, Nopachi, 1998). *In vivo*, reactive astrocytes are present within 24 hours of spinal cord injury (Hadley, Goshgarian, 1997) and eventually form a glial scar around the lesion site (Reier, Houle, 1988a). Thus, it is likely that astrocytes may be one of the cell types contributing to the increase in CSPG expression following spinal cord injury. Increases in CSPG expression have been co-localized with GFAP expression in the brain (Stichel et al., 1998). The results from our study, as well as preliminary findings by another group (Plant et al., 1998), show similar co-localization in the spinal cord. Activated microglia and macrophages may also contribute to injury-induced levels of CSPG expression. CSPG-IR was co-localized with these cells following small, discrete puncture wounds in the cerebellum (Levine, 1994) and a hemi-crush injury to the spinal cord (Fitch et al., 1996).

Microglia may also contribute to increased CSPG expression by secreting cytokines that stimulate astrocytic secretion of CSPGs (Asher et al., 1998; Geisert et al., 1998; Johnson et al., 1992; Koops et al., 1996; McKeon, Nopachi, 1998). It is likely that a variety of cell types contribute to CSPG production and that their relative contributions are dependent upon the injury model.

CSPGs Can Be Cleaved *In Vivo* by Chondroitinase ABC Application

We have shown that CSPGs can be cleaved *in vivo* by the exogenous application of the bacterial enzyme, chondroitinase ABC. To our knowledge, this is the first time that degradation of CSPGs with an exogenous enzyme has been shown in the spinal cord. The ability to enzymatically cleave CSPGs *in vivo* using chondroitinase ABC has important implications based upon several *in vitro* studies. These studies report a diminished inhibition following chondroitinase ABC degradation (Bovolenta et al., 1993; Brittis et al., 1992; Canning et al., 1996; McKeon et al., 1995; McKeon et al., 1991; Perris et al., 1996) and suggest that degradation of chondroitin sulfated glycosaminoglycan (CS GAG) chains without disruption of the protein core is sufficient to interfere with the growth inhibitory functions of CSPGs. Due to its site of action, this particular enzyme may have benefits over some other compounds. For example, β-xylosides block the attachment of GAGs to the protein core. This prevents the formation of intact CSPGs as well as other proteoglycans including heparan and keratan sulfate proteoglycans whose effects on growth may be distinctly different from CSPGs' (Chernoff, 1998; Dow et al., 1994; Dow et al., 1991; Halfter et al., 1997; Seo, Geisert, 1995). In addition, if the CS GAGs are responsible for the growth inhibitory function (Bovolenta et al., 1993; Brittis et al., 1992; Canning et al., 1996; McKeon et al., 1995; McKeon et al., 1991; Perris et al., 1996), then

chondroitinase ABC which selectively degrades CS GAGs would be more effective than other enzymes that cleave only the protein core (Chernoff, 1998).

Our results provide novel information about CSPG degradation, removal and production in the spinal cord. The lack of CSPG cleavage in saline controls, normal controls and contusion-injured-only spinal cords, suggests that endogenous enzymes are not active in the normal or injured adult rat spinal cord that are capable of cleaving CSPGs at the same site resulting in CSPG-IR. This makes chondroitinase ABC a useful enzyme for experimental studies because there is no confusion regarding the effects of endogenous versus applied enzyme. The decrease seen in CSPG cleavage at six days and its absence at eight days suggests two things. First, the applied chondroitinase ABC is no longer present or capable of cleaving CSPGs after six days. Secondly, the protein core which remains after cleavage with chondroitinase ABC is cleared from the injury site. If the cleaved CSPG molecules were not being removed, the extent of cleaved CSPGs would be expected to remain constant or increase over time. Instead, the extent of CSPG cleavage decreases such that at eight days it is non-existent. This lack of cleaved CSPGs reflects upon the exhaustion/removal of the exogenously applied enzyme and not the absence of CSPGs. CSPG-IR (which identifies cleaved and uncleaved CSPGs) shows a dense CSPG pattern that persists for up to forty days post-injury. This suggests that new CSPGs are being produced. If new CSPG molecules were not being produced, CSPG-IR would be expected to decrease as the cleaved CSPGs were removed.

These CSPG cleavage studies also provide information that may be important if chondroitinase ABC is studied for its therapeutic potential. A single application of chondroitinase ABC absorbed gelfoam cleaves CSPGs for a restricted time period. To be

effective in promoting regeneration, the delivery of chondroitinase ABC may need to be timed with a critical window for regeneration or modified to allow longer delivery. In addition to the restricted time frame, the cleavage seen with chondroitinase ABC is spatially limited with a gelfoam application. The greatest extent of CSPG cleavage seen was limited to a thin rim around the spinal cord. Cleaved CSPGs were never seen in the center of the spinal cord. The delivery of chondroitinase ABC may need to be modified to allow longer delivery in a more widespread fashion in order to assess its effects on axonal growth. Interesting approaches may include the use of pumps and/or gene delivery.

CSPG-IR Remains Elevated in the Host and Develops in the Fetal Graft Following Intraspinal Transplantation

Following intraspinal transplantation of fetal spinal cord, the host and graft appear to integrate well (Howland et al., 1996). The glial scar that is characteristic of spinal cord injuries is often reduced and, in some instances, absent following intraspinal transplantation (Houle, 1992). In cases where the glial scar is only partially disrupted, graft derived axons are reported to travel along the host-graft interface until they reach a break in the glial scar before passing into the host tissue (Houle, 1992). Reports such as these, in addition to studies correlating the presence of the glial scar with a lack of regeneration, suggest that a glial scar inhibits axonal growth (Reier et al., 1989; Reier, Houle, 1988a). The glial scar *in vivo* (Hall et al., 1991), as well as many of its cellular constituents *in vitro* (McKeon et al., 1995; McKeon et al., 1991; Rudge, Silver, 1990), have been shown to produce CSPGs. Thus, one might hypothesize that CSPGs along with the glial scar should be reduced around the lesion site following intraspinal transplantation. We found that this is not true. The increased expression of CSPGs, particularly around the fetal graft, correlates well with

CSPGs' putative role as a growth inhibitor because connections between the host and graft are limited (Jakeman, Reier, 1991). Most of the growth into and out of the graft is confined to within 4 mm of the host-graft interface (Jakeman, Reier, 1991). Even in the apparent absence of a glial scar, the host is intensely immunoreactive for CSPG. It is likely, however, that host astrocytes contribute to the production of CSPGs. The areas of CSPG-IR correlate with large areas of general gliosis identified by GFAP staining.

Our results also show that CSPG-IR develops in the intraspinal graft of fetal spinal cord tissue. The production of CSPGs within the graft is initiated within a few days of transplantation. Although CSPG-IR is relatively low at three days post-transplantation, it is greater than seen in the E14 spinal cord at the time of transplantation. CSPG expression is present in the central nervous system during fetal development (Landolt et al., 1995; Li et al., 1996; Milev et al., 1998b; Oakley et al., 1994; Oakley, Tosney, 1991; Pettway et al., 1996; Pettway et al., 1990). Thus, CSPGs in the grafted tissue may reflect some elements of normal development. It is also possible that CSPG expression is enhanced by the graft's proximity to injured adult tissue. Cells and/or molecules derived from the host may infiltrate and induce the graft to produce CSPGs. Astrocytes, microglia and macrophages from the host, all of which are capable of CSPG production (Canning et al., 1996; Fitch et al., 1996; Grierson et al., 1990; Kappler et al., 1997; Ramon-Cueto et al., 1998; Smith-Thomas et al., 1994; Zuo et al., 1998), may migrate into the graft and secrete CSPGs. In addition, inflammatory molecules from the host, such as TGF-β, may penetrate and trigger cells within the graft to produce CSPGs.

If CSPGs are growth inhibitory, the robust CSPG-IR at the host/graft border, at all time points studied, may inhibit axonal connectivity between the host and graft. Even

though grafted tissue is initially relatively low for CSPGs, host axons attempting to enter the graft would be confronted with a wall of host-derived CSPGs. Axons originating in the graft and attempting to enter the host, would also encounter this same host-derived wall of CSPGs. Studies by others in which DRG cells are transplanted into adult white matter also show a correlation between CSPGs and a lack of axonal growth (Davies et al., 1997). Transplanted DRG cells encapsulated by CSPGs in the host white matter did not extend processes in contrast to unencapsulated grafts that extended axons for long distances. In addition, developmental studies suggest that neurons in the fetal graft are likely to be inhibited by CSPGs. During development, embryonic neurons avoid areas and substrates that contain CSPGs (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Katoh-Semba et al., 1995; Oakley, Tosney, 1991; Perris, Johansson, 1987; Snow, Letourneau, 1992). In contrast to the host environment, the graft environment, at least initially, would provide axons in the graft with a relatively CSPG negative environment in which to grow. Previous studies have shown that internal neuronal processes and connections are abundant within intraspinal grafts (Grabowski et al., 1995). These processes must grow within the graft after transplantation because there are relatively few axons at the time of transplantation. We believe it is likely that this growth occurs before the graft becomes intensely CSPG-IR.

Conclusions

The immunoexpression of a group of putative inhibitory molecules, CSPGs, increases in the adult spinal cord as early as four days post-contusion and remains robust in the dorsal one-half to two-thirds of the spinal cord for as long as forty days. This robust CSPG pattern is not altered by intraspinal transplantation of fetal spinal cord tissue. In

fact, CSPG-IR increases over time within the intraspinal graft. The spatial and temporal immunocytochemical staining pattern of CSPGs in both the injured and injured plus transplanted spinal cords indicates that these molecules are present at the correct time and location to inhibit growth. The ability to enzymatically degrade these putative growth inhibitors *in vivo* is possible with chondroitinase ABC. These CSPG cleavage studies provide novel insights about CSPG production in the spinal cord and contribute valuable information for possible therapeutic applications in the future.

CHAPTER 3
ANALYSIS OF AGGRECAN CORE PROTEIN IN THE EMBRYONIC, ADULT, AND INJURED ADULT SPINAL CORD

Introduction

Aggrecan is a large proteoglycan that has been grouped with many different proteoglycan families based upon its physical characteristics (Doege et al., 1991; Hascall et al., 1998; Neame, Sandy, 1994). Its large core protein (210-350kD) has three globular domains and contains a hyaluronic acid binding region (HABR) in the globular 1 domain. Its HABR classifies aggrecan as an aggregating proteoglycan. Aggrecan is also considered a chondroitin sulfate proteoglycan (CSPG) due to the large number of chondroitin sulfate (CS) glycosaminoglycan (GAG) chains covalently attached to its core protein. In addition to CS GAG chains, aggrecan may also have keratan sulfate (KS) GAG chains. In this circumstance, aggrecan is also considered a keratan sulfate proteoglycan (KSPG). Aggrecan's inclusion in these three groups of proteoglycans illustrates the overlap of the evolving proteoglycan nomenclature and the need to investigate individual proteoglycans rather than proteoglycan groups.

Earlier studies have suggested that aggrecan or an aggrecan-like protein may exist in the central nervous system (CNS) (Asher et al., 1995; Bignami et al., 1993; Domowicz et al., 1995; Fryer et al., 1992; Li et al., 1996; Milev et al., 1998b). However, there are no conclusive studies showing aggrecan protein expression in the spinal cord. Definitive identification and analysis of the expression of aggrecan in spinal cord is necessary to

begin to clarify the potential influences of this proteoglycan in the CNS. Although there is no direct evidence of the function of either the CS GAG or core protein of aggrecan in the CNS, earlier studies have examined the influences of groups of proteoglycans, including CSPGs, to which aggrecan belongs. When studied as a family, CSPGs appear to inhibit neurite growth in cell culture (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Dou, Levine, 1995; Perris, Johansson, 1987; Rudge, Silver, 1990; Snow, Letourneau, 1992; Yamada et al., 1997) and are correlated with a lack of regeneration following injury in the CNS *in vivo* (Fitch, Silver, 1997; Gates et al., 1996; Lemons et al., 1999) (also described in Chapter 2). The growth inhibitory effects of CSPGs *in vitro* can be diminished following selective degradation of the CS GAG chains by chondroitinase ABC (Dow et al., 1994; Risling et al., 1993; Snow et al., 1990; Zuo et al., 1998). These studies suggest that aggrecan may inhibit growth through its CS GAG chains. This appears to be the case for another specific CSPG, brevican, that, like aggrecan, belongs to the group of aggregating proteoglycans (Yamada et al., 1997). In most of the published work, however, the presence and abundance of CSPGs has been determined using antibodies against CS and the identity of the proteoglycans (PGs) which carry the CS has not been established. The antibodies which have been used, therefore, would be expected to detect any PGs with a CS GAG chain including aggrecan, brevican, neurocan, versican, phosphacan, decorin, biglycan, and NG2. All of these PGs have been reported to exist in the CNS (Engel et al., 1996; Margolis et al., 1996; Meyer et al., 1996; Milev et al., 1998b; Rauch et al., 1992; Schmalfeldt et al., 1998; Stichel et al., 1995; Yamada et al., 1997; Yamaguchi, 1996).

In order to begin to determine the relative importance of the different CSPGs following injury to the spinal cord, we have initiated a study evaluating aggrecan, in addition to several other hyaluronan-binding proteoglycans, in the developing, adult and injured adult rat spinal cord. In these studies, we have used western blot analysis and a panel of mono-specific antibodies against aggrecan, neurocan, brevican and versican. In addition, we have used neoepitope antisera against specific degradation products of aggrecan. Two of the neoepitopes studied (NITEGE, TFKEEE) are generated by the action of aggrecanase (ADAMTS)-like activity on hyaluronan-binding PGs (Sandy et al., 1995). A third neoepitope studied, FVDIPEN, results from the action of metalloproteinases (MMPs) or cathepsin B (CatB) on aggrecan (Boyd et al., 1990; Lark et al., 1995).

Our data show that aggrecan is a normal constituent of the spinal cord in the embryo and throughout life. Western blot analysis shows that the full length aggrecan protein core (350kD) is present in embryonic day 14 (E14), postnatal day 1 (P1) and adult rat spinal cord. Aggrecan and brevican fragments that result from the action of aggrecanase (ADAMTS)-like activity are present. There is also evidence for MMP/cathepsin B degraded aggrecan. There is no striking difference between the aggrecan products seen during development and adulthood. Additionally, the spectrum of these products does not appear to be markedly influenced by spinal cord injury. Using western blot analysis, we also show that cultured astrocytes, derived from adult spinal cord, can produce aggrecan.

Methods

Hemisection Spinal Cord Injury

Adult Long Evans rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (35mg/kg for females and 40 mg/kg for males). All animals were given the antibiotic, penicillin G procaine (Phoenix Pharmaceutical Inc., St. Joseph, MO), sub-cutaneously for seven days, beginning on the day of surgery (30,000U/250g). Surgical procedures were carried out under sterile conditions and on a warming pad. The low thoracic spinal cord was exposed by a laminectomy and the dura mater was slit. Iridectomy scissors were used to make three cuts in the spinal cord. Two unilateral hemisections 2mm apart were made and the third cut connected the medial aspects of the hemisections. Gentle aspiration was used to lift out the tissue isolated by the cut and any remaining tissue to make a complete hemisection. If the edges of the dura matter remained intact following hemisection injury, the dura matter was sutured. The muscle and skin were closed in layers. Rats recovered in a veterinary intensive care unit and were re-hydrated immediately post-surgery with subcutaneous injections of 3 cc of saline. Rats' bladders were manually expressed twice daily until bladder function returned.

Tissue Preparation

Animals (three timed-pregnant, seven postnatal day 1, five normal adult, nine spinal cord injured-adult rats) were deeply anesthetized with an overdose of sodium pentobarbital (>50mg/kg). An ovariohysterectomy procedure was performed on time-pregnant animals at fourteen days post-conception. The intact uterus was removed and placed upon ice. The embryonic day 14 (E14) spinal cords were kept cold while the spinal

cords were carefully dissected and the dura matter removed under a microscope. The spinal cords of postnatal day 1 (P1) rats were similarly removed under a dissecting microscope. The spinal cords of normal adults or spinal cord injured adults were removed following a laminectomy procedure. A large laminectomy was made from T10 through L2. Twenty-five mm of the exposed spinal cord was quickly removed by severing the roots and cutting the spinal cord. The injured spinal cords were blocked into 5, 5 mm pieces with the center piece containing the lesion epicenter. Each 5 mm piece roughly approximated one spinal segment. All harvested spinal cords (E14, P1, adult, injured-adult) were immediately rinsed with cold phosphate buffered saline (PBS) and placed into cold "total proteinase inhibitor cocktail" (Boehringer Mannheim, Chicago, IL) with 5mM iodoacetic acid, 0.1mM 4-(2-aminoethyl)benzenesulphonyl flouride (AESBF), 1% CHAPs, 1μg/ml pepstatin A and 50mM sodium acetate (from Sigma, St. Louis, MO). The tissue was then quickly cut into 5mm pieces while in the proteinase inhibitor solution, blotted dry, weighed and placed into 1.5 ml tubes with fifteen volumes (1.6μl/mg tissue) of cold extractant solution (proteinase inhibitor solution containing 4M guanidine hydrochloride, pH, 7.6). Each sample was homogenized, using a pre-chilled "tissue tearer" (from Biospec Products, Inc.), for 3-5 seconds and extracted for sixteen hours at 4° C. The samples were clarified by centrifugation at 15,000 g for one hour at 4° C. A floating layer of insoluble material (myelin) was removed. The clear extracts were precipitated overnight with 3 volumes of cold ethanol/5mM sodium acetate at 0° C. Precipitated protein and proteoglycans were collected by centrifugation for one hour at 12,000g at 4° C. Ethanol was removed and the pellet spinal cord tissue was digested with

0.9U purified Chondroitinase ABC (Sigma)/ 1ml buffer (50mM sodium acetate, 50mM Tris hydrochloride, 10mM EDTA, pH, 8) for three hours at 37° C.

Protein concentration was determined using a modification of a published method (Bradford, 1976). Briefly, in a 96 well plate, samples were assayed with 250 μls of Bradford reagent (Sigma) and a plate reader (programmed to read at 595nm). A dilution series (0μg- 10μg) of bovine serum albumin (Sigma) were also assayed used to generate a standard curve. The protein concentration of each sample was calculated based upon the standard curve.

Cell Cultures

Purified astrocytes were prepared from adult rat cerebral cortices and spinal cord using a modification of a published protocol (Smith, Hale, 1997). The meningeal tissue was removed and tissue was diced into 1-2mm pieces and then placed in a calcium and magnesium-free buffer (MEM-CMF) containing 0.025% trypsin and 2mM EDTA for 5 minutes at 37° C with gentle shaking. The tissue was then treated with DNase (50 UG) for 5 minutes under the same conditions. An equal volume of DMEM (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum was added to MEM-CMF buffer. The tissue was spun down, media aspirated and the tissue was re-suspended in fresh DMEM with 10% fetal bovine serum. Cells were disassociated, passed through a cell strainer (Fisher), and plated into polylysine-coated 75 cm^2 tissue culture flasks at a density of 2.0×10^7 cells/flask. After the cells reached confluence (approximately two to three weeks), astrocytes were purified by vigorous shaking and treated with 10^{-5} M cytosine arabinoside for two days. Astrocytes were plated onto 12.5 cm^2 filtered flasks (1×10^6 cells/flask) and grown in media with N2 supplement (Gibco) for ten days. Astrocyte

conditioned medium was acetone precipitated at -20° C overnight and protein pellets were collected by centrifugation at 12,000 rpm. Each protein pellet was re-suspended in 100 µls of chondroitinase buffer containing 10µls (0.9U) of purified chondroitinase ABC (Sigma) and incubated for 3 hours at 37° C.

Electrophoresis

Gels were loaded on a protein basis. Chondroitinased-digested samples were mixed with 15µl of sample buffer (Novex 2X sample buffer containing 800mM DL-Dithiothreitol and 3M urea) and heated at 100° C for 5 minutes. The samples were loaded on a 10 well, 1.5mm thick, 4-12% SDS-PAGE gradient gel (Novex, San Diego, CA). See Blue pre-stained molecular weight markers (Novex) and biotinylated broad range molecular weight markers (Bio-Rad, Hercules, CA) were used as standards. Purified aggrecan from rat chondrosarcoma (RCS) digested with chondroitinase ABC, was used a positive control (Morgelin et al., 1988; Paulsson et al., 1987). Purified aggrecan from RCS contains full length aggrecan and several of its naturally occurring degraditive products. Aggrecan degraditive products from RCS are similar to those degraditive products typically found in cartilage (Sandy et al., 1995). The degraditive products in the CNS, however, may vary in size if enzymes in the CNS are different than those existing in cartilage. The gels were run at 125 V for eighty-five minutes in electrode buffer (25mM Tris base, 192mM glycine, 0.1% SDS).

Transfer and Immunoblotting

Gels that were processed for the presence of aggrecan were transferred onto a 0.45 micron nitrocellulose membrane in a Bio-Rad Transfer Unit filled with transfer buffer

(25mM Tris base, 192mM glycine and 20% methanol). These gels were transferred with 100 V for one hour at room temperature or 30 V overnight on ice. Gels that were processed for the presence of brevican were also transferred onto a 0.45 micron nitrocellulose membrane using a transfer buffer that did not contain methanol (25mM Tris base, 192mM glycine) with 150mA for 2 hours. Following transfer, all membranes were rinsed with 20mM Tris buffered saline containing 0.5% triton, 137mM sodium chloride at a pH, 7.6 (TBS-T) for five minutes and blocked in 5% non-fat dry milk (Carnation) in TBS-T. The membranes were incubated for forty-five minutes with one of several primary antibodies (anti-ATEGQV, TYKHRL, NITEGE, FVDIPEN, TFKEEE) diluted in TBS-T containing 1% non-fat dry milk (each primary was diluted at 1:3,000 except for FVDIPEN which was diluted at 1:1,000). These antibodies have been described previously (Lark et al., 1995; Sandy et al., 1998; Sandy et al., 1995) and are described in more detail below. Membranes were washed with TBS-T quickly two times and then three times for ten minutes each. Membranes were then incubated with goat anti-rabbit, peroxidase labeled antibodies (Vector, 1:5,000) in TBS-T containing 1% non-fat dry milk for thirty minutes. The portion of the nitrocellulose membrane that contained biotinylated markers was cut out and run separately from the portion of the membrane that contained spinal cord tissue. We have found that the strepavidin-horseradish peroxidase (HRP) used to label the biotinylated molecular weight markers can non-specifically bind to lanes containing spinal cord tissue. Thus, the portion of the membrane with biotinylated markers was cut and separately incubated with strepavidin-horseradish peroxidase (HRP) (Vector, 1:5,000) for thirty minutes. Two negative controls were done: 1) the membrane was not incubated with the primary antibody and 2) the membrane was incubated with the primary antibody

that had been pre-incubated with aggrecan (10μl of antibody with of 0.1mg of aggrecan dissolved in 100μl distilled water). All membranes were rinsed in TBS-T as described earlier and incubated with a chemiluminescent marker, ECL (Amersham, Arlington Heights, IL) for one minute. Film exposure times ranged from 15 seconds to 3 minutes to generate suitable images.

Primary Antibodies

Aggrecan-specific antibodies (ATEGQV and TYKHRL) and neoepitope antisera (NITEGE, TFKEEE, and FVDIPEN) have been previously described (Lark et al., 1995; Sandy et al., 1998; Sandy et al., 1995). The epitopes recognized by these antibodies are presented diagrammatically in Figure 3-1. The ATEGQV and TYKHRL antisera recognize epitopes in the G1 and G3 domains of aggrecan, respectively. Therefore, they react with full length aggrecan or degraditive products bearing the G1 or G3 domain alone. The specific peptide recognized by ATEGQV in the G1 domain (CATEGQVRVNSIYQDKVSLP) and by TYKHRL in the G3 domain (CTYKHRLQRTMRPTRRSRPSMAH) are not present in the core protein of any other sequenced PG. TFKEEE detects the neoepitope generated by aggrecanase-dependent cleavage of the Glu1459-Gly1460 bond of rat aggrecan (Sandy et al., 1995). NITEGE detects the neo-epitope generated by aggrecanase-dependent cleavage of the Glu373-Ala374 bond of rat aggrecan (Lark et al., 1995; Sandy et al., 1995). FVDIPEN detects the neo-epitope generated by MMP or CatB-dependent cleavage of the Asn341-Phe342 bond of rat aggrecan (Boyd et al., 1990; Lark et al., 1995).

The brevican monoclonal antisera (RB18; generously provided by Dr. Yamaguchi) was raised against the 80kD C-terminal fragment of brevican and, therefore, detects both

the full length (145kD) and the aggrecanase (ADAMTS)-generated C-terminal fragment (80kDa) (Yamada et al., 1997; Yamaguchi, 1996).

The two different neurocan antisera used (1F6 and 1D1; Hybridoma Bank, Iowa City, Iowa) specifically recognize the N-terminal and C-terminal halves of the molecule which are generated by proteolysis near the middle of the core protein (Meyer et al., 1995; Olsson et al., 1996).

Results

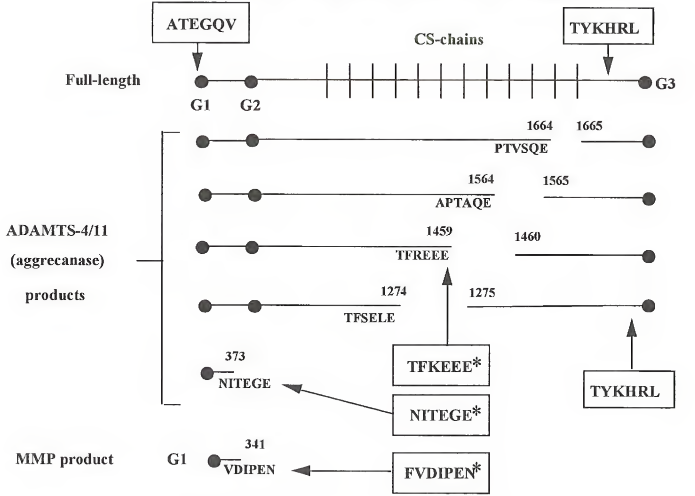
Our results show that aggrecan is present in the embryonic, postnatal, adult and injured spinal cord. Two aggrecan-specific antibodies, ATEGQV and TYKHRL, detected full length aggrecan as well as many of aggrecan's degraditive products in embryonic, postnatal and adult spinal cord. Many of the aggrecan degraditive products were seen in injured spinal cord tissue at one and two weeks post-injury. Aggrecan degraditive fragments were also identified by aggrecan specific neo-epitope antisera (TFKEEE, NITEGE and FVDIPEN). In addition, we show that cultured astrocytes from adult brain and spinal cord can produce and secrete aggrecan into their surrounding media.

ATEGQV and TYKHRL Identifies Aggrecan and Aggrecan Degraditive Products in the Spinal Cord

Aggrecan was detected in the normal, adult rat spinal cord using western blot analysis and aggrecan specific antibodies, ATEGQV and TYKHRL. These antisera have previously been shown to recognize aggrecan (full length and many degraditive fragments) from rat, human, dog, rabbit and bovine cartilages (Sandy et al., 1995). These antibodies were generated against peptide sequences from aggrecan that are not found in

Figure 3-1. Aggrecan antibody binding. Illustration of location of five aggrecan epitopes and some aggrecanase digestion sites. Each antibody is boxed and the neo-epitope antibodies are also marked by an asterisk. Two of the antibodies, ATEGQV and THKYRL, can label full length aggrecan and aggrecan degraditive products. Three neo-epitope antibodies, NITEGE, FVDIPEN and TFKEEE label sites that are exposed after enzymatic cleavage.

Antibody recognition sites on rat aggrecan



Residue numbers are showm for C-terminal neoepitope antisera *

Figure 3-2. Western blot analysis of aggrecan, brevican and neurocan in spinal cord tissue. (A) Aggrecan is identified by the ATEGQV antibody in purified aggrecan (lane 1), injured spinal cord tissue harvested at one week post-injury, rostral (lane 2) and caudal (lane 3) to the site of injury, injured spinal cord tissue harvested at two weeks post-injury, rostral (lane 4) and caudal (lane 5) to the site of injury, E14 spinal cord (lane 6), P1 spinal cord (lane 7) and uninjured adult spinal cord (lane 8). The 350kD band is referred to as band A, 250kD as B, 220kD as C, ~170kD as D, 135kD as E, 60, 55, 52 and 50kD bands as band F. Some of the aggrecan degraditive fragments are identified by neo-epitope anti-sera in normal adult spinal cord tissue such as: NITEGE positive 60kD band (lane 9), FVDIPEN positive 52kD band (lane 10) and TKFEEE positive 220kD band (and other smaller fragments also) (lane 11). RB18 labels 145 and 80kD brevican positive bands in normal spinal cord (lane 11) and P1 spinal cord (lane 13). RB18 did not detect brevican in E14 spinal cord (lane 12) or purified aggrecan (lane 14).

(B) Aggrecan is identified by TYKHRL antisera in purified aggrecan (lane 1), injured spinal cord tissue harvested at one week post-injury, rostral (lane 2) and caudal (lane 3) to the site of injury, injured spinal cord tissue harvested at two weeks post-injury, rostral (lane 4) and caudal (lane 5) to the site of injury, E14 spinal cord (lane 6), P1 spinal cord (lane 7) and uninjured adult spinal cord (lane 8). 1F6 (lane 9) and 1D1 (lane 10) antisera label 130kD and 150kD neurocan positive bands in normal spinal cord, respectively.

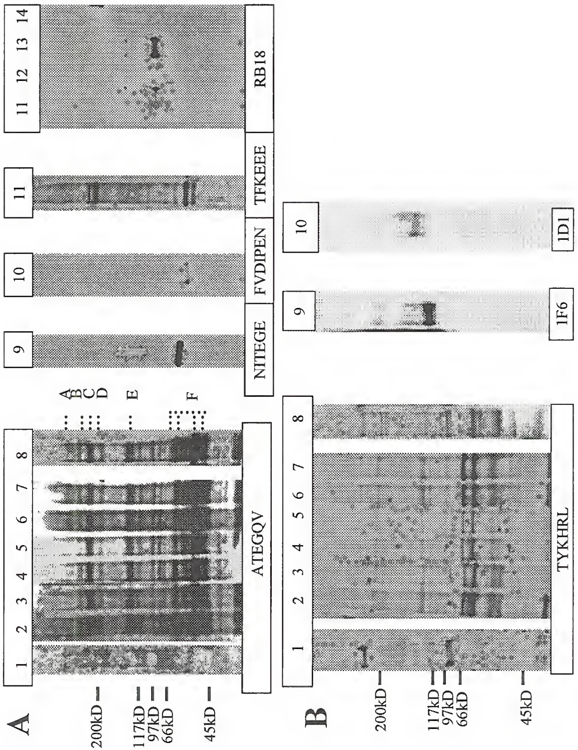
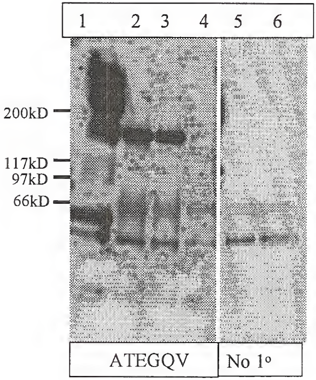

A
200kD
117kD
97kD
66kD
45kD
1 2 3 4 5 6 7 8
A B C D E F
ATEGQV
9
NITEGE
10
FVDIPEN
11
TFKEEE
11 12 13 14
RB18
B
200kD
117kD
97kD
66kD
45kD
1 2 3 4 5 6 7 8
TYKHRL
9
1F6
10
1D1

Figure 3-3. ATEGQV positive bands in astrocyte conditioned media. ATEGQV positive bands (220kD and ~170kD) are present in purified aggrecan (lane 1), spinal cord astrocyte conditioned media (lane 2) and brain astrocyte conditioned media (lane 3). These two bands are similar to ATEGQV positive bands C and D in spinal cord tissue (Figure 3-2). Two small faint bands are seen in non-conditioned media (lane 4) and in spinal cord and brain astrocyte conditioned media without primary antibody (lane 5 and 6, respectively).



1 2 3 4 5 6
200kD
117kD
97kD
66kD
ATEGQV
No 1°

any other sequenced proteoglycan. These antisera do not appear to detect other aggregating proteoglycans such as brevican (Figure 3-2 A), neurocan (Figure 3-2 B) and versican (Sandy, Kenagy and Wright, unpublished results). The one possible exception is the E band (~135kD)on the ATEGQV blots which appears to be similar in size to one of the neurocan IF6 positive bands (130kD) (Figure3-2). It is unlikely that ATEGQV is cross-reacting with neurocan based upon peptide sequences (i.e. there is not a 5 residue stretch, or greater, in neurocan that is identical to any portion of the antiserum antigen used to generate ATEGQV). To further validate their specificity, ATEGQV and THYKRL would have been incubated on membranes that contained lanes of purified neurocan and brevican. However, purified sources of these proteoglycans are not available to our laboratory.

ATEGQV and TYKHRL positive bands were abundant in embryonic, postnatal and mature spinal cord extracts (Figure 3-2A and B, lanes 6-8). The banding patterns were similar for each respective antibody across the time points studied. Both of these antibodies detected full length aggrecan (350kD) and many G1 (ATEGQV) and G3 (TYKHRL) containing aggrecan fragments. Many of these aggrecan degraditive fragments were also found in purified aggrecan from RCS. Aggrecan fragments are continually generated in cartilage *in vivo* and in cultured chondrocytes stimulated with cytokines. These fragments have been shown to be the result of aggrecan degradation by aggrecanase (an ADAMS), other MMPs and possibly CatB (Fosang et al., 1992; Grumet et al., 1996; Hughes et al., 1995; Rauch et al., 1997; Sandy et al., 1992; Sandy et al., 1998; Sandy, Lark, 1999; Sandy et al., 1991; Sandy et al., 1995). The ATEGQV and TYKHRL

antibodies identified many degraditive fragments in the spinal cord, some of which were clearly different from those identified previously in cartilage or RCS.

Many of the ATEGQV positive bands are discussed in more detail below. To simplify the description of the ATEGQV positive bands, they are referred to as follows: the 350kD band (full length aggrecan core protein) as A, 250kD band as B, 220kD band as C, an approximately 170kD band as D, ~135kD band as E, and the ~60kD, ~55kD, ~52kD and ~50kD bands as F. Bands B, C, D, E and F are truncated forms which retain the N-terminus G1 domain but have different C-termini. The specificity of this antiserum for aggrecan G1 domain in spinal extracts was established as follows: ATEGQV bands were not detected if: 1) the primary antibody was not applied during immunoblotting or 2) the primary antisera was pre-incubated with purified aggrecan prior to application to the membrane.

Degraditive Products Are Identified by Neo-Epitope Antisera

Aggrecan neo-epitope antisera (TFKEEE, NITEGE, FVDIPEN) identified G1 containing aggrecan degraditive products present in spinal cord tissue. The neo-epitope antisera confirmed many of the smaller ATEGQV positive bands to be aggrecan specific degraditive products. For example, the TFKEEE and NITEGE neo-epitope antisera label two different sized aggrecan degraditive products (220 and 60kD, respectively) that are known to result from aggrecanase digestion (Figure 3-2A). These two products appear to correspond to the ATEGQV-positive products identified as band C and some of band F (Figure 3-2). The 60kD NITEGE positive species is a common product of aggrecan degradation in cartilage and rat chondrosarcoma extracts (Lark et al., 1995; Sandy et al., 1998; Sandy et al., 1995). The 220kD TFKEEE positive species has also been identified in

cartilage and rat chondrosarcoma extracts (Sandy et al., 1998). The other smaller TFKEEE positive bands in spinal cord tissue have not been commonly observed in cartilage. These smaller bands could be: 1) degraditive fragments of aggrecan that have been cleaved at other sites in addition to the one that is TFKEEE positive (Figure 3-1) and 2) degraditive fragments of versican. TFKEEE antisera can label degraditive versican fragments in addition to aggrecan degraditive fragments. However, based upon these proteoglycans' amino acid sequences, only aggrecan can produce a 220kD TFKEEE positive degraditive product. Thus, this 220kD TFKEEE positive band is most likely an aggrecan degraditive product. In addition, the FVDIPEN neo-epitope antisera labeled a G1 containing aggrecan degraditive product (52kD) that results from MMP or CatB degradation. This 52kD band apparently detects one component of the bands (band F) in the ATEGQV stained membrane (Figure 3-2).

Effects of Spinal Cord Injury upon Aggrecan

Many aggrecan positive bands were detected in the injured spinal cord using ATEGQV and TYKHRL antibodies. The staining pattern appeared similar at the level of the lesion as well as rostral and caudal to the injury site (Figure 3-2) These bands were very similar to those seen in embryonic, postnatal and adult spinal cord tissue with one exception. The full length (350kD) aggrecan core protein (band A) was not detected in injured spinal cord tissue harvested at one week post-hemisection. However, a faint 350kD band (equivalent to ATEGQV band A) was detected with TYKHRL antisera in injured spinal cord tissue harvested at two weeks post-hemisection. ATEGQV bands B, C, D, E and F were consistently present in the injured spinal cord tissue at one and two weeks post-injury. Bands B, C, D, E and F appeared similar to those in the normal spinal cord.

Cultured Astrocytes Produce Aggrecan

ATEGQV positive bands were present in astrocyte conditioned media (Figure 3-3). The expression of aggrecan appeared similar in astrocytes derived from brain and spinal cord of adult rats. Although full length aggrecan was not detected, 220kD and ~170kD bands were seen in the lanes containing brain and spinal cord derived astrocyte conditioned medium (Figure 3-3). These two bands (220kD and ~170kD) correspond to bands C and D seen in normal and injured adult spinal cord using the ATEGQV antibody (Figure 3-2A). Both bands are also seen in lanes containing purified aggrecan from RCS. The 220kD and ~170kD bands were not present in the lane containing non-conditioned media. Two more lightly stained bands (approximately 60 and 50kD) were detected in: 1) astrocyte conditioned media , 2) non-conditioned astrocyte media and 3) astrocyte conditioned media without application of ATEGQV anti-sera, suggesting that these bands do not represent aggrecan degraditive fragments made by cultured astrocytes. It is possible, however, that these bands could be a combination of specific and non-specific labeling because ATEGQV positive bands are detected at similar molecular weights in lanes containing purified aggrecan. These 60 and 50kD bands are also very similar to some of the ATEGQV positive bands that are in band F in the normal and injured spinal (Figure 3-2A).

Other Aggregating Proteoglycans Are Present in the Spinal Cord

Brevican and neurocan, two other aggregating proteoglycans, were detected in normal spinal cord. A specific brevican antibody (RB18) identified two bands (145kD and 80kD) in the adult and P1 spinal cord. Brevican was not detected in E14 spinal cord or in purified aggrecan from RCS. The two bands in the adult appeared more robust than those seen in the P1 spinal cord (Figure 3-2). Neurocan was identified in the spinal cord with two

antibodies (1D1 and 1F6) generated against the C- and N-terminal epitope of the molecule (Meyer et al., 1995; Rauch et al., 1992) (Figure 3-2). The 1D1 antibody appeared to label a 150kD band in adult spinal cord tissue. The 1F6 antibody appeared to label 130kD band in normal, adult spinal cord tissue.

Discussion

These studies show that aggrecan is present in the embryonic, neonatal, adult and injured adult rat spinal cord. These results are consistent with other studies that have shown aggrecan is present in the brain (Li et al., 1996; Milev et al., 1998b). In addition, these results are consistent with previous studies that describe an "aggrecan-like" protein in the adult dog, rat and hamster spinal cord (Asher et al., 1995; Fryer et al., 1992; Kalb, Hockfield, 1990; Zaremba et al., 1989). Our studies are also unique from previous work in four ways: 1) the use of two aggrecan-specific antibodies (ATEGQV and TYKHRL) that identify full length aggrecan and many aggrecan degraditive products on Western blots, 2) aggrecan (full length core protein and many degraditive products) is identified in embryonic, postnatal, adult and injured spinal cord, 3) the use of neo-epitope aggrecan antibodies (NITEGE, FVDIPEN and TFKEEE) that identify specific degraditive fragments of aggrecan and 4) identification of aggrecan in astrocyte conditioned media.

The Presence of Aggrecan in the Developing and Mature CNS

Full length aggrecan core protein (350kD) and several smaller bands, presumably degraditive aggrecan products, were detected in embryonic, neonatal and adult rat spinal cord with ATEGQV and TYKHRL antibodies. The pattern of aggrecan bands did not appear to differ between in E14, P1 and adult spinal cord. These results suggest that

aggrecan expression and degradation are similar at these times. The consistent pattern of aggrecan expression in the immature and mature spinal cord differs from previous reports of increasing aggrecan expression in brain. Using slot blot radioimmunoassays, Milev et al. (1998) have shown an increase in aggrecan expression in the brain from E14 to five months of age. It is possible that the expression patterns of aggrecan may differ between the brain and the spinal cord. It is also possible that methodological differences in aggrecan antibodies and protein analysis techniques (Western blot and slot blot radioimmunoassay) in these studies may account for this apparent discrepancy. For example, Western blot analysis with two aggrecan-specific antibodies (ATEGQV and TYKHRL) produce an array of bands that reveal both full length aggrecan and aggrecan degraditive products. However, slot blot analysis (that was done with a different aggrecan antibody) produces one signal that is not capable of distinguishing full length aggrecan from aggrecan degraditive fragments (Milev et al., 1998). The ability to analyze several aggrecan bands versus one aggrecan signal, in addition to differences in aggrecan antibody selection, could contribute to these conflicting reports in aggrecan expression with age.

The influences of aggrecan in the CNS are not known, however its influences can be hypothesized based upon effects of other proteoglycans in the same chondroitin sulfate or aggregating proteoglycan groups. For example, aggrecan could be one of the CSPGs that appears to repulse migrating cells and act as a guidance molecule during development (Oakley et al., 1994; Oakley, Tosney, 1991). Also, aggrecan could be one of the CSPGs that can inhibit neurite growth in cell culture (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992) and is correlated with a lack of regeneration following spinal cord injury (Fitch, Silver, 1997; Lemons et al., 1999) (also described in Chapter 2). In addition,

aggrecan's core protein may influence the matrix of the spinal cord by binding specific molecules such as tenascin (Milev et al., 1998a; Rauch et al., 1997)or TGF-β (Andres et al., 1992; Boyd et al., 1990; Hildebrand et al., 1994), similar to other proteoglycans. Aggrecan also may contribute to the structure and/or integrity of spinal cord tissue as it does in cartilage. The high negative charge of aggrecan's CS GAG chains allows it to bind water and is thought to contribute to the turgor and integrity of cartilage (Sandy et al., 1996). The identification of aggrecan in the spinal cord opens the door to explore its possible influences in the spinal cord.

Aggrecan Degraditive Products Are Present in the Spinal Cord

ATEGQV and TYKHRL antisera labeled full length aggrecan and smaller bands that are presumed to be aggrecan degraditive products. The likelihood that these smaller positive bands are aggrecan degraditive products was supported by the use of neo-epitope antisera (TFKEEE, FVDIPEN and NITEGE) that identify specific aggrecan degraditive fragments. For example, two ATEGQV positive bands (220 and 60kD) in the spinal cord and in aggrecan from RCS were also identified by TFKEEE and NITEGE staining, respectively. These two antibodies label two G1 containing aggrecan degraditive products generated by aggrecanase (Figure3-1) (Sandy et al., 1998). Aggrecanase is a MMP that is likely to belong to the ADAMTS family (Arner et al., 1999). Aggrecanase is present in cartilage and cleaves aggrecan, brevican and versican at the Glu^{373}-Ala^{374} bond and other bonds as well (Sandy et al., 1995). This is the first time, to our knowledge, that aggrecanase or aggrecanase-like digestion-products have been shown in the CNS. These results suggest the presence and activity of aggrecanase or an aggrecanase-like enzyme in the spinal cord.

A 52kD ATEGQV positive band that was detected in spinal cord tissue and in purified aggrecan also was identified by the FVDIPEN antibody. FVDIPEN labels a G1 containing 52kD aggrecan degraditive fragment resulting from MMP or CatB digestion of aggrecan (Andres et al., 1992; Boyd et al., 1990; Hughes et al., 1995). Metalloproteinases are abundant in cartilage and regularly cleave aggrecan (Bonassar et al., 1997; Bonassar et al., 1996). Many metalloproteinases also are present in the spinal cord (Chandler et al., 1997; Kieseier et al., 1998; Pagenstecher et al., 1998) and should be capable of cleaving aggrecan. These results suggest that some of the same enzymes that cleave aggrecan in cartilage appear to be active in the spinal cord.

Some of the ATEGQV and TYKHRL positive bands in the spinal cord and in aggrecan from RCS are different from one another. This is not surprising because it is likely that some different enzymes may exist in the spinal cord versus cartilage. The unique profile of enzymes in these two systems could account for some of the ATEGQV and TYKHRL bands in spinal cord tissue that are not found in aggrecan from RCS and vice versa.

Aggrecan Is Present Following Spinal Cord Injury

Many smaller ATEGQV and TYKHRL positive bands were detected in injured spinal cord tissue at one and two weeks following surgery in areas immediately rostral and caudal to the lesion epicenter (Figure 3-2). These aggrecan degraditive fragments in the injured spinal cord tissue were similar to those found in uninjured spinal cord tissue. These results suggest that enzymes that cleave aggrecan in the injured spinal cord are similar to those in the uninjured spinal cord and/or that aggrecan fragments were not cleared from the spinal cord.

Interestingly, full length aggrecan core protein (350kD) was not detected at one week post-hemisection, but was detected at two weeks post-hemisection. The inability to detect full length aggrecan at one week post-injury suggests: 1) that full length aggrecan is not produced in the spinal cord lesion area at one week following hemisection in sufficient quantities to be detected using these methods or 2) that aggrecan is continually produced following injury but is rapidly cleaved by an injury-induced increase in enzymatic activity that precludes full length aggrecan from being detected at one week. It is not uncommon for proteins to breakdown post spinal cord injury due to direct damage or increased production of enzymes. The possibility that the degradation of aggrecan may be enhanced following injury is supported by previous studies that report an increased expression of many enzymes that cleave aggrecan following injury. For example, expression of MMP-2 and 9 increases following injury *in vivo* and by activated microglia and astrocytes *in vitro* (Gottschall et al., 1995; Pagenstecher et al., 1998; Romanic et al., 1998; Rosenberg, 1995). Thus, the increase in enzymes, such as MMPs, may contribute to the degradation of aggrecan. Due to the faint 350kD band present in normal spinal cord tissue, it seems possible that an increase in the activity of aggrecan cleaving enzymes may be capable of reducing full length aggrecan to non-detectable levels. A faint 350kD TYKHRL positive band (full length aggrecan) was detected at two weeks post-surgery. This suggests that full length aggrecan may have been below detection levels at one week post-injury and is returning to detectable levels. The subthreshold levels of aggrecan could be due to: 1) injury-induced levels of enzymatic activity may be decreasing and/or 2) aggrecan production was temporarily decreased following injury.

Aggrecan may Contribute to the Lack of Significant Growth following CNS injury

CSPGs have previously been shown to inhibit neurite growth *in vitro* (Snow et al., 1996; Snow, Letourneau, 1992; Snow et al., 1991) and have been correlated with a lack of regrowth following spinal cord injury *in vivo* (Fitch, Silver, 1997; Lemons et al., 1999) (also described in Chapter 2). The presence of aggrecan in the spinal cord qualifies this molecule as one of CSPGs that may contribute to the failure of regeneration in the CNS. Experiments are underway in our laboratory to evaluate the effects of aggrecan upon axonal growth *in vivo*.

Although full length aggrecan is not detected at one week post-injury, many degraditive fragments are present. It is possible that some of the aggrecan fragments may be capable of inhibiting growth. For example, the CS GAG chains on aggrecan (that are present on the larger degraditive fragments such as band B, C and D) may inhibit growth. Several studies have demonstrated the ability of CS GAG chains to contribute to CSPGs growth inhibitory effects (Brittis et al., 1992; Dow et al., 1994; Pettway et al., 1990; Risling et al., 1993). In addition, it appears as though some CSPGs, such as NG2, can inhibit growth via their protein core (Dou, Levine, 1994). Perhaps a specific portion of the core protein is responsible for this inhibition. It is possible that a particular portion of aggrecan's core protein is critical to its putative influence upon neurite growth. Even in the unlikely event that full length aggrecan is needed to influence growth, the 350kD band is detected at two weeks post-injury. Thus, it would be present to inhibit growth at later time points.

Astrocytes Can Secrete CSPGs Including Aggrecan

Our results show that cultured astrocytes can secrete aggrecan into their surrounding media. Previous studies have shown that cultured astrocytes could secrete CSPGs (Canning et al., 1996; Johnson-Green et al., 1991; McKeon et al., 1991) and some preliminary studies suggested that astrocytes can produce at least three specific proteoglycans: neurocan, phosphacan, and NG2 (Asher et al., 1998; McKeon, Nopachi, 1998). However, our results are the first to show that cultured astrocytes can produce aggrecan (Figure 3-3). Although full length aggrecan was not detected in the astrocyte conditioned media, two smaller aggrecan products (220 and ~170kD) were detected. Similar bands appeared in the lane containing purified aggrecan, suggesting that these bands may be aggrecan degraditive fragments. The 220kD and ~170kD ATEGQV positive bands were similar to bands C and D in spinal cord tissue (Figure 3-2). These results suggest that astrocytes can produce two of the aggrecan bands consistently seen in embryonic, postnatal, adult and injured adult spinal cord. It is possible that astrocytes produce full length aggrecan and perhaps aggrecan degraditive enzymes that generate these smaller ATEGQV positive bands. The lack of other ATEGQV bands (e.g., similar to bands B, E and F) in astrocyte conditioned media suggests a lack of other enzymatic activities in this cell culture assay that appear to be present in spinal cord tissue. The production of aggrecan by cultured astrocytes supports the hypothesis that the co-localization of CSPG-immunoreactivity (IR) and glial fibrillary acid protein (GFAP)-IR suggests that astrocytes secrete CSPGs (Engel et al., 1996; Gates et al., 1996; Pindzola et al., 1993; Yamada et al., 1997). Furthermore, the ability of cultured astrocytes to secrete aggrecan suggests that aggrecan: 1) is likely to be present in (although not limited to) the

extracellular matrix of the CNS and 2) may be one of the CSPGs present in the glial scar that contribute to the non-permissiveness of the glial scar (McKeon et al., 1995; McKeon et al., 1991).

Other Aggregating Proteoglycans Are Present in the Spinal Cord

Our results show that brevican and neurocan, two aggregating proteoglycans, are present in the spinal cord. The intensity of two brevican positive bands (145kD and 80kD) was greatest in the adult spinal cord, less intense in the P1 spinal cord and not detected in the E14 SC (Figure 3-2A). These results suggest that the expression of brevican increases with age in the spinal cord. Previously, brevican has not been documented in the spinal cord, however it has been shown in the brain (Milev et al., 1998b; Seidenbecher et al., 1998; Yamada et al., 1997; Yamaguchi, 1996). Its expression patterns in the developing and adult brain are similar to our results in the spinal cord. In the brain, brevican expression in the adult is greater than the neonate (Milev et al., 1998b). Thus, it appears that brevican expression increases with age in the brain and in the spinal cord.

The 1D1 and 1F6 neurocan antibodies identified neurocan in the adult spinal cord. Previously, neurocan has only been shown in the adult and embryonic brain and in the embryonic spinal cord (Engel et al., 1996; Katoh et al., 1998; Meyer et al., 1996). Full length neurocan core protein (245kD) was not seen in the adult spinal cord with either of these antibodies. This is consistent with other studies that show that the 245kD protein is not present in significant amounts in the adult brain (Rauch et al., 1991). Smaller 1D1 and 1F6 positive bands (130 and 150kD, respectively) were found in adult spinal cord and are similar to those found in the adult brain (Rauch et al., 1991; Rauch et al., 1992). The distinct temporal expression patterns of specific aggregating proteoglycans (such as

brevican, neurocan and aggrecan) suggest that each of these proteoglycans may have different influences upon cell migration and neurite growth in the immature and mature CNS.

In summary, these studies identified aggrecan in the embryonic, adult and injured spinal cord. Aggrecan may be one of the proteoglycans that can inhibit growth (Snow et al., 1996; Snow, Letourneau, 1992), serve as a developmental guidance molecule (Fichard et al., 1991; Landolt et al., 1995; Oakley et al., 1994; Oakley, Tosney, 1991; Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990) or possibly contribute to the lack of regeneration following CNS (Davies et al., 1997; Fitch, Silver, 1997; Gates et al., 1996; Lemons et al., 1999) (also described in Chapter 2). Aggrecan can be secreted by cultured astrocytes and therefore, aggrecan may be one of the proteoglycans present in the glial scar that can inhibit growth (McKeon et al., 1995; McKeon et al., 1991).

CHAPTER 4
AGGRECAN, A PROTEOGLYCAN PRESENT IN THE CENTRAL NERVOUS SYSTEM, INFLUENCES AXONAL GROWTH *IN VIVO*

Introduction

Aggrecan is an aggregating proteoglycan that is found in a variety of tissues (Doege et al., 1987; Doege et al., 1991; Hardingham, Bayliss, 1990; Hardingham, Fosang, 1992; Juul et al., 1991; Pettway et al., 1996; Roughley, Lee, 1994; Sandy et al., 1995; Sandy et al., 1996) including the central nervous system (Li et al., 1996; Milev et al., 1998b) (also described in Chapter 3). Aggregating proteoglycans are characterized by a hyaluronic acid (HA) binding region that enables them to attach to HA and form large, aggregating complexes (Hascall et al., 1998; Mow, Rosenwasser, 1988; Neame, Sandy, 1994; Sandy et al., 1996). Like many aggregating proteoglycans (e.g., versican, neurocan, brevican), aggrecan has chondroitin sulfate (CS) glysoaminoglycan (GAG) chains attached to its core protein (Hardingham, Fosang, 1992; Kjellen, Lindahl, 1991a; Roughley, Lee, 1994; Ruoslahti, 1988). Proteoglycans (PGs) that contain at least one CS GAG chain are referred to as chondroitin sulfate proteoglycans (CSPGs).

CSPGs as a group have been shown to serve as developmental guidance molecules (Fichard et al., 1991; Landolt et al., 1995; Oakley et al., 1994; Oakley, Tosney, 1991; Perris et al., 1991; Pettway et al., 1996; Pettway et al., 1990) and/or inhibit neurite growth *in vitro* (Carri et al., 1988; Carson et al., 1992; Dou, Levine, 1994; Oohira et al., 1991; Perris et al., 1996; Rudge, Silver, 1990; Snow et al., 1996; Snow et al., 1990; Snow,

Letourneau, 1992; Yamada et al., 1997). In these earlier PG studies, individual CSPG members were not identified. Based upon its inclusion in the CSPG family, aggrecan could be one of the CSPGs that appear to have inhibitory influences. In fact, the CSPGs used in some of these earlier studies have recently been identified as aggrecan (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992; personal communication, Diane Snow). The growth inhibitory effects of some CSPGs appear to be diminished following digestion by the bacterial enzyme, chondroitinase ABC (Friedlander et al., 1999; McKeon et al., 1995; Snow et al., 1990; Yamada et al., 1997; Snow et al., 1990). Chondroitinase ABC cleaves CS GAG chains on CSPGs, leaving the core protein intact with short sugar stubs. These studies suggest that the CS GAG chains may be critical to the growth inhibitory effects of CSPGs. However, this is not always the case. The inhibitory influences of some CSPGs, such as NG2, persist following digestion with chondroitinase ABC (Dou, Levine, 1994). Thus, the growth inhibiting mechanism may differ among various CSPGs. The increased expression of CSPGs following injury to the adult CNS (Fitch, Silver, 1997; Gates et al., 1996; Lemons et al., 1999) (also described in Chapter 2), in combination with their effects *in vitro* and during development, suggests that CSPGs may contribute to the lack of regeneration following injury. In Chapter 3, we have shown that aggrecan is one of the CSPGs present in the normal and injured spinal cord. The aims of this study were to begin to address aggrecan's possible growth inhibitory influences by determining if: 1) if aggrecan could inhibit growth and 2) if its putative inhibitory capacity could be diminished by chondroitinase ABC digestion. In order to address these aims, we developed an *in vivo* growth assay that is used in the following studies to determine aggrecan's effect on regeneration.

The *in vivo* growth assay provides a robust growth arena that can be challenged with the addition of aggrecan, or any other proteoglycan. This assay is a cross between cell culture and *in vivo* studies. The *in vivo* growth assay is able to maintain injured neurons in their normal environment while presenting their axons to a cell culture-like controlled environment, within which axonal growth can be assessed. Results from this assay show that aggrecan can inhibit axonal growth. Furthermore, chondroitinase ABC did not significantly diminish aggrecan's axonal growth inhibitory effects in this assay. Thus, aggrecan, which we have previously identified in the injured spinal cord (described in Chapter 3) may contribute to the lack of regeneration in the injured spinal cord.

Methods

For all surgical procedures, adult Long Evans rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (35mg/kg for females and 40 mg/kg for males). All animals were given the antibiotic, penicillin G procaine (Phoenix Pharmaceutical Inc., St. Joseph, MO), sub-cutaneously for seven days, beginning on the day of surgery (0.1cc (30,000U)/250g). All surgical procedures were carried out under aseptic conditions and on a warming pad. Rats recovered in veterinary intensive care units and were re-hydrated with subcutaneous injections of 3 cc of saline. Rats' bladders were manually expressed twice daily until bladder function returned.

Surgical Lesion and Matrix Placement

The low thoracic spinal cord was exposed by a laminectomy and the dura mater was slit. Prior to formation of a left hemi-aspiration cavity, two left dorsal rootlets were

severed close to the dorsal root entry zone and gently pulled back from the hemi-aspiration site to protect them from further damage. The hemisection lesion was made with iridectomy scissors and aspiration as follows: using iridectomy scissors, two unilateral cuts, approximately 2mm apart, were made and a third cut connected the medial aspects of the hemisections. Gentle aspiration was used to lift out the tissue isolated by the cuts and to lift any remaining tissue so that it could be easily cut to make a complete hemisection cavity. Using sterile, cooled pipette tips, 3-4 μls of one of four matrix suspensions (see below) were placed into each lesion cavity. Two previously severed dorsal rootlets were immediately placed into the matrix suspension and an additional 1-2 μls of the matrix suspension were placed over the rootlets. After the matrix had gelled *in vivo*, the dura matter was sutured (when feasible) and the site covered with dura film. The muscle and skin were closed in layers.

Matrix Suspensions

As indicated above, one of four types of matrices was placed in the lesion cavities. One matrix suspension consisted of matrigel-only. The specific type of matrigel used in these studies is reduced basement membrane matrigel (Collaborative Biomedical Products) that supports nerve regeneration (Madison et al., 1985) and cell differentiation (Kleinman et al., 1986). A second matrix suspension was composed of matrigel plus aggrecan (300 μg aggrecan/1ml matrigel). This concentration of aggrecan (300 μg aggrecan/1ml matrigel) was within the range of aggrecan concentrations used in other studies (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992). In the experiments described in this paper, aggrecan was isolated from rat chondrosarcoma (RCS) (Morgelin et al., 1988;

Paulsson et al., 1987). The third matrix group was composed of matrigel loaded with chondroitinased aggrecan (referred to as the core protein (CP) of aggrecan) at 30μg/ml matrigel. Briefly, the CP was isolated by digesting aggrecan with chondroitinase ABC, running this sample on a Superose 12 column and pooling the CP using a Dionex detector. Under these conditions, short sugar CS stubs as well as N- and O-linked oligosaccharides remain on the core protein. The fourth and final matrix consisted of aggrecan's CS-GAG chains added to matrigel at 270μg/ml. Briefly, the CS GAG chains were collected from aggrecan (purified from RCS) that had been digested with papain and precipitated with cold ethanol/5mM sodium acetate. The quantities of CP and CS GAG chains that were added to the matrigel were based upon their percent composition in intact aggrecan. The CP represents 10% and the CS GAGs represents 90% of aggrecan's molecular weight.

Perfusions and Basic Histology

Rats were anesthetized with an overdose of sodium pentobarbital (> 50mg/kg) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (pH, 7.4). The spinal cord lesion/implant sites were blocked and equilibrated in a 30% sucrose phosphate buffer (pH, 7.4). Fourteen-micrometer serial sections were cut on a cryostat and thaw mounted onto chrom-alum, poly-L-lysine subbed slides or charged slides (Fisher). Every ninth and tenth section were processed with cresyl violet (cresyl violet with acetate, Sigma) and myelin (Eriochrome Cyanine R, Fluka, New York) stains for basic histology. The remaining sections were processed for immunocytochemistry.

Immunocytochemistry

Tissue sections were processed for visualization of neurofilament (anti-neurofilament from Zymed, San Fransciso) and p75 (anti-p75 from Promega, Wisconsin) proteins using immunohistochemistry techniques. Briefly, sections were rinsed in phosphate buffered saline (PBS) with 0.4% Triton (Sigma) and 1% horse serum (PBS-T-HS) for tissue treated with the monoclonal neurofilament antibody or in PBS-T containing 1% goat serum (PBS-T-GS) for tissue treated with the polyclonal p75 antibody. Spinal cord sections were blocked for one hour with PBS-T containing 10% of the appropriate animal serum. Tissue was incubated with either the neurofilament or p75 primary antibodies overnight in the cold room (neurofilament at 1:3200 in PBS-T-HS and p75 at 1:333 in PBS-T-GS). The next day at room temperature, spinal cord sections were rinsed before and after a one hour incubation with an appropriate secondary antibody (horse anti-mouse at 1:100 in PBS-T-HS from Vector Laboratories, Burlingame, CA and goat anti-rabbit, rat and mouse adsorbed at 1:100 in PBS-T-GS from Biogenics, San Ramone, CA). The tissue was subsequently incubated with a tertiary complex (ABC Elite from Vector Laboratories), rinsed and reacted with 3,3'diaminobenzyadine tetrahydrochloride (DAB, from Sigma). Negative controls were not incubated with primary antibody. The specimens were gradually dehydrated, de-fatted and coverslipped.

Inclusion Criteria

Twenty six animals were selected for this study. Five animals were included in the matrigel group, eight animals in the aggrecan group, seven animals in the CP group and six animals in the CS GAG group. Animals selected for this study had to meet the

following inclusion criterion based upon terminal histological analysis. First, hemisection lesions had to be filled with a matrigel based matrices. Second, a group of dorsal root fibers had to be seen entering each of the matrix-filled hemisections. Because two dorsal rootlets were placed together at the time of surgery, these fibers appeared to fuse in most animals. If more than one group of fibers were seen entering any of the matrices, the animal was not included in the study.

Quantification of Neurite Growth

A modification of the procedure described by Howland et al., (1995) was used to compare the extent of growth into the various matrices. A reticle grid (10x10 squares, each $0.05mm^2$, at a magnification of 20x) was used to calculate the percent area within the grid that contained neurites. The first grid was placed at the tip of the dorsal rootlet in the matrix. The number of the grid squares containing neurites was then counted, recorded and referred to as the percent area containing fibers for Grid or Box A. The grid was then placed immediately rostral and adjacent to the edge of Box A. The number of grid squares containing neurites in this grid (Box B) was also counted, recorded and referred to as percent area containing fibers for Box B (Figure 4-1B). In addition, the total number of squares that contained neurites in Box A and Box B was recorded as the percent area containing fibers for Box (A+B). This quantification procedure was done on the tissue section with the greatest extent of neurofilament staining from each animal in each group. Thus, each animal and group is represented by their greatest ingrowth. All quantification was blinded and the group identity of the specimens was revealed only after the

completion of the quantification procedure. The percent area containing ingrowth was compared across groups with a one way ANOVA using the Bonferroni method.

Results

Growth into Matrigel-Only Filled Lesions

At one week post-surgery, a few neurofilament positive fibers were seen in the matrigel-filled lesions (Figure 4-2A). The fibers were located near the dorsal rootlets and some appeared to extend from the edge of the severed rootlets. The neurofilament profiles in the matrigel at this time were thin and scarce. Fibers were rarely found in the rostral aspect of the matrigel-filled lesion.

At fourteen days post-surgery, the matrigel-filled lesion was filled with numerous neurofilament profiles (Figure 4-2C). Fibers were distributed throughout the matrigel matrix. The distribution and number of fibers in the matrigel-filled lesions appeared to greatly increase from seven to fourteen days post-surgery.

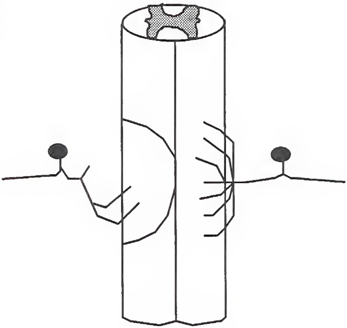
In some animals (n=4), dorsal rootlets were not placed into matrigel-filled lesions. Neurofilament profiles were limited in number and in distribution in the matrices of these animals (described in Chapter 5). The majority of the fibers were seen at the host-matrigel interface. The pattern of growth was not as robust at those matrigel-loaded matrices with dorsal rootlet placement.

Growth into Aggrecan-Loaded Matrix is Greatly Reduced

At one week post-surgery, very few (if any) neurofilament profiles were seen within the aggrecan-loaded matrigel matrix. In fact, in some animals, many large swollen end

Figure 4-1. Diagram of *in vivo* assay (A) and growth quantification method (B). A spinal cord hemisection is filled with one of four matrices: 1) matrigel-only, 2) intact aggrecan-loaded matrigel, 3) aggrecan core protein-loaded matrigel and 4) aggrecan CS GAG loaded matrigel. Two severed dorsal rootlets are placed into the matrix (A). Using a microscope, an eye reticle grid was laid down over the matrigel at the place of dorsal rootlet entry (called Box A). The number of squares containing fibers is counted and recorded as percent area containing growth for Box A. The eye reticle grid is laid down again at a place immediately adjacent to Box A. The number of squares containing fibers in this grid (called Box B) is counted and recorded as percent area containing growth for Box B.

A



B

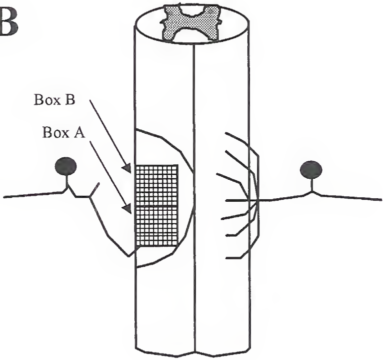


Figure 4-2. Representative photomicrographs of longitudinal spinal cord sections through a matrix-filled hemisection stained for neurofilament protein (A-F). A limited number and extent of neurofilament positive fibers are present in the matrigel-only matrix at seven days post-surgery (A). At this same time, there are hardly any fibers present in the aggrecan-loaded matrigel matrix (B). At fourteen days post-surgery, neurofilament positive fibers are abundant and fill the matrigel-only filled lesion cavity (C). Fibers in the aggrecan-loaded matrigel are not nearly as abundant or widespread at this same time point (D). Neurofilament positive profiles are present in the CS GAG loaded matrigel matrix at two weeks (E), but they are not as abundant at the time-matched matrigel-only control (C). Fibers are also present in the CP loaded matrigel group, however they do not fill the lesion as well as matrigel-only controls. dr=dorsal rootlet, open arrows mark host/matrigel interface, dark arrows in A label axons extending into matrigel, dark arrows in F label lateral edge of CP loaded matrigel

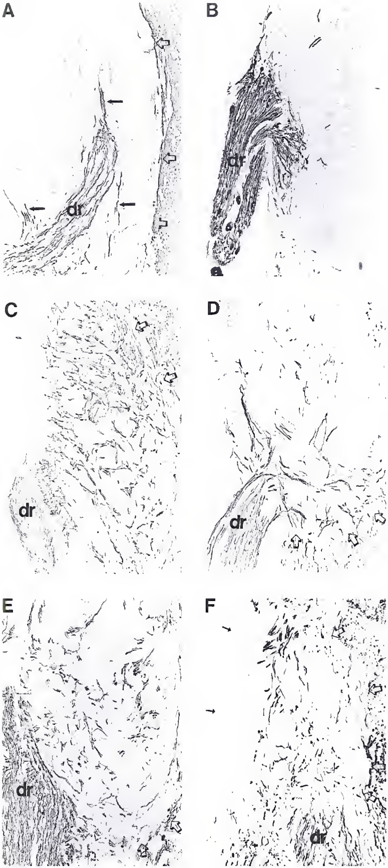
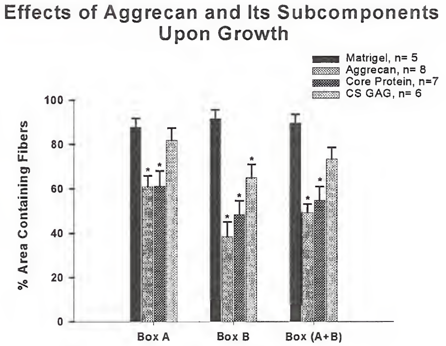

A
B
C
D
E
F
dr
dr
dr
dr
dr
dr

Figure 4-3. Quantification of growth into four different matrices at two weeks post-surgery. A vertical bar graph compares the mean value of each group and also indicates which groups are statistically significant from matrigel-only matrices (A). In Box A (reticle grid closest to the dorsal rootlet), Box B (reticle grid rostral to Box A) and Box (A+B), the greatest growth was found in the matrigel-only matrix. The matrigel-matrix served as a positive control. Neurite growth was significantly reduced in the aggrecan-loaded matrix in Box A, Box B and Box (A+B) compared to the positive control. Neurite growth was also significantly and consistently reduced in the CP-loaded matrigel matrices. Neurite growth was also less, but not always significantly less, in the CS GAG loaded matrigel matrices. Growth into the CS GAG loaded matrices was not as inhibited as the aggrecan and CP matrices.

A point plot graph shows the raw data points for each animal included in the study (B). The left panel shows data from Box A, the middle panel shows data from Box B and the right panel shows data from Box (A+B).

A



* significantly different from Matrigel, p< 0.05

B

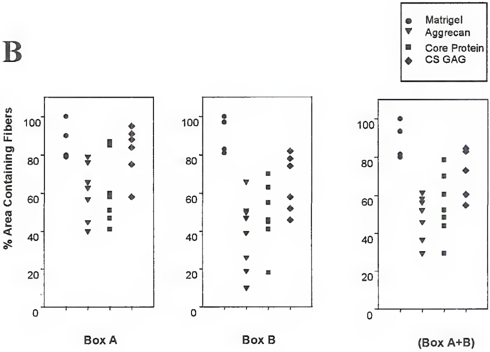
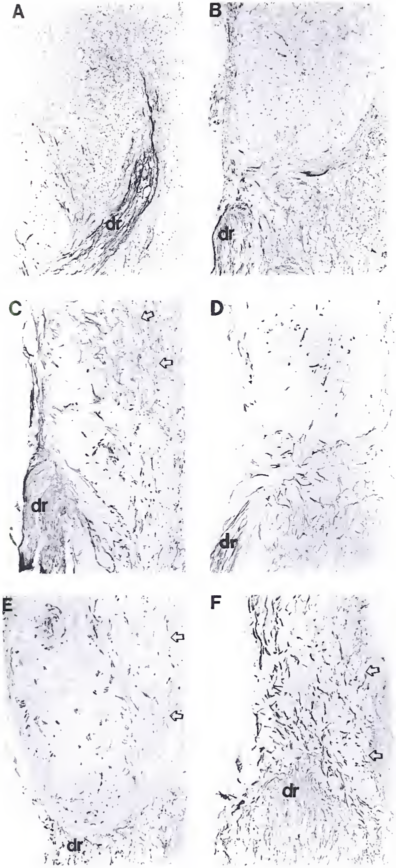


Figure 4-4. Representative photomicrographs of longitudinal spinal cord sections processed for immunohistochemistry with a p75 antibody to label Schwann cells in matrix-filled hemisections (A-F). At seven days post-surgery, a few Schwann cells are seen near the dorsal rootlet placed into matrigel-only matrices (A). At this same time, a few lightly-stained Schwann cells are present in the aggrecan-loaded matrigel (B). At fourteen days post-surgery, there are Schwann cells lightly scattered throughout matrigel-only matrices (C), aggrecan-loaded matrices (D), CS GAG loaded matrices (E), CP-loaded matrices (F). dr=dorsal rootlet, open arrows mark the host/matrix interface.


A
B
C
D
E
F
dr
dr
dr
dr
dr
dr

bulbs similar to those described by Cajal (Ramon y Cajal, 1991) were apparent close to the severed edge of the dorsal rootlet in the matrix (Figure 4-2B, also described in Chapter 5).

At two weeks post-surgery, some neurofilament profiles were present in the aggrecan-loaded matrigel. These profiles, however, did not appear as widespread or numerous as in the time-matched matrigel-only matrices. In the aggrecan-loaded matrices most of the fibers in the matrigel were close to the dorsal rootlets, along the caudal/lateral edge of the matrices and/or close to the caudal host/matrix interfaces. Few fibers were seen in the middle of the aggrecan-loaded matrix (Figure 4-2D). Some fibers were in the rostral aspect of the matrigel, very close to the rostral host/matrigel interface (not shown). A few terminal clubs were also seen in the aggrecan-loaded groups at two weeks, but these clubs were smaller, less numerous and more distant from the edge of the dorsal rootlet (not shown). Overall, in the aggrecan-loaded matrices the pattern of neurofilament profiles was dramatically less than that seen in time-matched matrigel-only matrices.

Neurofilament Profiles in Matrices Loaded with the CP and the CS GAG of Aggrecan

The effects of CP and CS GAG loaded matrices were evaluated only at two weeks post-surgery. At this time, growth into the matrices loaded with aggrecan CP was scarce and unevenly scattered in the matrix (Figure 4-2F). The neurofilament positive profiles in the CP-loaded matrices generally were less robust and less widespread than time matched matrigel-only matrices (Figure 4-2C). However, the distribution of fibers in Box B of CP-loaded matrices appeared greater than Box B in aggrecan-loaded matrices (Figure 4-2D).

At two weeks post-surgery, neurofilament profiles were scattered throughout the matrices loaded with CS GAG. Their distribution, however, did not appear as dense as seen in matrigel-only groups. In the CS GAG-loaded matrices, fibers generally appeared more abundant near the dorsal rootlets than the fibers in the more rostral portion (Box B) of the matrix (Figure 4-2F). Fibers in the CS GAG-loaded matrices also appeared to be less abundant than the matrigel-only matrices (Figure 4-2C) but more widespread than the aggrecan-loaded matrices. (Figure 4-2D).

Two Week Matrigel-Only Group Serves as Positive Control

At one week post-surgery, growth into matrigel-only matrices was not significantly robust to qualitatively appreciate a noticeable difference between matrigel-only and aggrecan-loaded matrices. However, by two weeks, there was extensive and consistent ingrowth of fibers into the matrigel-only matrices. This high growth arena allowed the matrigel-only group at two weeks to serve as a strong positive control. Thus, the CP and CS GAG loaded matrices were only analyzed at two weeks post-surgery. There appeared to be a qualitative difference at this time between matrigel-only and other matrices. Thus, growth at fourteen days post-surgery was quantitatively analyzed and compared in matrigel-only matrices and other matrigel based matrices loaded with: 1) intact aggrecan, 2) the CP of aggrecan (chondroitinase ABC digested aggrecan) and 3) the CS GAG of aggrecan (Figure 4-3).

Quantification of Neurofilament Profiles in Four Different Matrices

Using a reticle grid (see methods), the percent area containing fibers was quantified in matrices containing matrigel-only and matrigel loaded with: 1) intact aggrecan, 2) CP of

aggrecan and 3) CS GAG of aggrecan. The percent area that contained fibers was averaged across animals in each group and defined as the percent area containing fibers for each of these groups (Figure 4-3). In Box A (adjacent to the dorsal rootlet), fibers in the matrigel-only were present, on average, in 87.8% of the area in this box. In contrast, fibers in the aggrecan-loaded, CP-loaded and CS-GAG loaded matrices were present in 61.1%, 61.2% and 81.8% of the area in Box A, respectively. A one way ANOVA, using the Bonferonni's method, showed that the percent area of growth in Box A of the intact aggrecan and aggrecan CP groups was significantly less than the positive control (matrigel-only). The percent area of growth was somewhat less in the CS GAG group than the positive control, but this difference did not reach statistical significance ($p>0.05$).

In Box B, (adjacent and rostral to Box A), 91.6% of the area contained fibers in the positive control, whereas 38.5%, 48.2% and 65% of the area contained fibers in aggrecan, CS GAG and CP-loaded matrigel, respectively. The percent area of growth for Box B in each of the three experimental groups was significantly less than the matrigel-only group.

The area encompassed by both Box A and Box B nearly filled the entire matrix-filled hemisection. Therefore, the total number of squares containing growth in Box (A+B) was an estimate of the percent area of the entire matrix-filled hemisection that contained fibers. In the positive control group, fibers were present in 89.7% of the total squares in Box (A+B). Fibers in aggrecan, CP and CS GAG-loaded matrices were found in 49.2%, 54.8% and 73.4% of the area in Box (A+B), respectively. The values for the intact aggrecan and the CP group were significantly less than matrigel-only. The percent area containing growth in the CS GAG group was generally less than the positive control but did not achieve statistical significance ($p>0.05$).

There was a statistically significant difference between the percent area of growth in the intact aggrecan and CS GAG group in Box A, Box B and Box (A+B). There was also a significant difference between the percent area of growth in the CS GAG loaded matrices and in the CP loaded matrices in Box A, Box B and Box (A+B) ($p<0.05$). However, the percent area containing fibers in the intact aggrecan group and CP group in Box A, Box B or Box (A+B) was not statistically significant ($p>0.05$). These results suggest the influences of intact aggrecan and CP-loaded matrigel were somewhat similar whereas the influences of intact aggrecan and CS GAG loaded matrigel were significantly different.

Schwann Cells Migrate into Matrices

It is possible that Schwann cells may migrate into the lesion/matrix site and influence to axonal ingrowth. Thus, the presence of Schwann cells in all four types of matrices were examined on slides adjacent to those that had been stained for neurofilament protein. Schwann cells were identified by p75 immunostaining and by the presence of a nucleus at a higher power. At one week post-surgery, Schwann cells were limited in number and lightly scattered in matrigel and aggrecan-loaded matrices (Figure 2-2A,B). At two weeks post-surgery, Schwann cells appeared slightly more numerous but were still scantily distributed throughout all types of matrices at two weeks post-surgery (Figure 4-3C,D,E,F). When p75 stained tissue was compared to NF stained tissue on an adjacent slide, it appeared as though Schwann cells were generally present in a similar vicinity as fibers. However, in some cases, fibers were not present in the same area as Schwann cells and Schwann cells were not always present in the same area as fibers (not shown).

Discussion

We chose to evaluate the effects of aggrecan in the *in vivo* growth assay for three reasons: 1) aggrecan has been identified in the spinal cord (described in Chapter 3) and brain (Li et al., 1996; Milev et al., 1998b), 2) a CSPG that has been identified as aggrecan can inhibit neurite outgrowth *in vitro* (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992) and 3) purified aggrecan could be obtained in sufficient quantities for the *in vivo* growth assay.

Aggrecan Inhibits Axonal Growth of Adult Neurons

At seven days post-surgery, aggrecan's putative growth inhibitory influence is suggested by the presence of many terminal clubs on dorsal rootlet axons that were placed into aggrecan-loaded matrices (Figure 2-2, B). Terminal clubs are considered an anatomical hallmark of regeneration failure and were described by Cajal in the early 1900's as a sign that a neuron was incapable of overcoming an obstacle or create a new path (Cajal, 1991). A few terminal clubs were also seen in the aggrecan-loaded groups at two weeks, but these clubs were smaller, less numerous and more distant from the edge of the dorsal rootlet (not shown). At fourteen days post-surgery, the extent of fibers in aggrecan-loaded matrices appeared dramatically less than matrigel-only matrices at fourteen days post-surgery (Figure 4-2). Quantitatively, the distribution of fibers at two weeks post-surgery in the aggrecan-loaded matrices was significantly less than time matched matrigel-only matrices (Figure 4-4). These results suggest that aggrecan can inhibit axonal growth of adult neurons *in vivo*.

Our results are consistent with and extend previous cell culture studies that suggest that CSPGs, later identified as aggrecan, can inhibit neurite growth (Snow et al., 1996; Snow et al., 1990; Snow, Letourneau, 1992: personal communication, Diane Snow). These *in vitro* studies showed that neurite outgrowth of embryonic neurons (from chick dorsal root ganglion, retinal ganglion and rat forebrain) was decreased by aggrecan bound to the surface of a cell culture dish or nitrocellulose paper. Our results extend beyond these studies in that they suggest that aggrecan can also inhibit axonal growth of non-disassociated, adult neurons in a three-dimensional assay.

Chondroitinase ABC Digestion of Aggrecan Does Not Significantly Diminish Growth Inhibitory Effects of Aggrecan in the *In Vivo* Growth Assay

Axonal growth into CP-loaded matrices is significantly decreased compared to positive controls (Figure 4-3). Interestingly, there is not a significant difference between the growth into CP-loaded and intact aggrecan-loaded matrices. These results suggest that the CP of aggrecan can inhibit axonal growth to an extent that is not significantly different from intact aggrecan. The ability of the CP of aggrecan to significantly inhibit axonal growth in this assay is particularly impressive due to the limited amount of aggrecan core protein present in the matrix (0.015μg core protein/5μls of matrigel per hemisection lesion) compared to intact aggrecan (1.5μg aggrecan/5μls of matrigel). These results suggest that: 1) small amounts of aggrecan CP can strongly influence growth and 2) that a primary inhibitory component of aggrecan may reside in the core protein. It is, however, possible that the short CS sugar stubs or the N– or O-linked oligosaccharides that remain on the core protein following chondroitinase ABC digestion may contribute to this influence (the CP was isolated using chondroitinase ABC digestion, see methods).

Despite this possibility, these studies suggest chondroitinase ABC digestion does not significantly diminish aggrecan's growth inhibitory effect in this assay. This is in contrast to previous cell culture studies that suggest that chondroitinase ABC digestion does diminish aggrecan's growth inhibitory effects (Snow et al., 1990; personal communication with Diane Snow). There are several methodological differences between these studies that may account for the apparent disparity. For example, previous studies tested the effects of chondroitinased aggrecan upon disassociated embryonic chick dorsal root ganglion in a two dimensional cell culture assay. However, studies described in this chapter test the effects of chondroitinased aggrecan upon non-disassociated adult rat neurons *in vivo* in a three dimensional assay. It is possible that neuronal differences in age and species (Snow, Letourneau, 1992) as well as the presentation of aggrecan (Snow et al., 1996) in a two dimensional versus three dimensional system may account for apparent conflicting reports.

The studies in this chapter are not the first to suggest that a growth inhibitory component of a specific CSPG may lie in the CP. Another CSPG, NG2, also retains its inhibitory influences upon neurite growth following chondroitinase ABC digestion (Dou, Levine, 1994; Doucette et al., 1994). Furthermore, it has been suggested that NG2 inhibits growth via a cell surface receptor that is linked, either directly of indirectly to intracellular messenger systems (Dou, Levine, 1997). It is possible that aggrecan core protein may work through a receptor system also.

Axonal Growth into CS GAG loaded aggrecan

In the CS GAG loaded matrices, the percent area containing fibers was significantly less from controls in Box B but not in Box (A+B) or in Box A. These results suggest that

although the CS GAG of aggrecan appears to inhibit axonal growth into the rostral aspect of the matrix, the CS GAG did not significantly influence growth in the caudal aspect of the matrices nor did it significantly influence growth overall in the matrices (Box A+B). Together, these results suggest that there may be a differences in the rostral and caudal aspect of the matrices. It is likely that the caudal aspect (Box A) of matrices contains mostly dorsal root axons and could contain some spinal cord axons. However, the rostral aspect (Box B) of matrices may contain long fibers from the dorsal rootlet and/or axons from the spinal cord. The ability of host spinal cord axons to extend into matrigel matrices is suggested, but not conclusively proven, by the presence of fibers in matrigel-loaded matrices without dorsal rootlet placement (described in Chapter 5). Fibers that are present in these matrices without dorsal rootlets are limited in number and are lightly scattered compared to matrigel-loaded matrices with dorsal rootlet placement. Therefore, it is likely that the many of the fibers seen in the matrices with dorsal rootlet placement originate from the dorsal root ganglion .

It is possible that neurons from different sources (dorsal root ganglion, raphe nucleus, noradrenergic, etc...) may be uniquely influenced by matrigel, aggrecan, CP or CS GAG containing matrices. It is also possible that intact aggrecan, the CP of aggrecan and/or the CS GAG of aggrecan may effectively inhibit more distant axonal growth. This possibility could account for the consistent decrease in growth into Box B for all experimental groups, assuming Box B is mostly comprised of distant dorsal root fibers. It is possible that the CS GAG of aggrecan may selectively inhibit distant growth of dorsal root fibers due to its significant growth inhibition in Box B. Regardless of fiber

composition or distance issues, aggrecan inhibits axonal growth of adult neurons in this *in vivo* assay.

Schwann Cells Migrate into Matrices

Schwann cells are present in all four types of matrices (Figure 4-4A-F). The results from these studies do not conclusively demonstrate that aggrecan, the CP of aggrecan or the CS GAG of aggrecan inhibit Schwann cell migration. In many cases, axons are often found in the general vicinity as Schwann cells on adjacent slides, suggesting that Schwann cells may guide axonal growth. However, this is not always true. There are instances where axons are seen in Schwann cell free areas and vice versa. The ability to interpret spatial relationships between immuno-localization on adjacent slides is limited. Ideally, double immunofluorescence would have been used to more accurately determine spatial relationships between neurofilament protein and p75 protein, however, immunofluorescence techniques are not successful in matrigel. These results, therefore, are not conclusive regarding spatial relationships between axons and Schwann cells, however, they do suggest that aggrecan, the CP of aggrecan and the CS GAG of aggrecan do not completely inhibit Schwann cell migration.

Possible Mechanisms of Aggrecan Inhibition

The mechanisms through which CSPGs influence growth are not well known. However, these studies, in combination with other studies, suggest several possible mechanisms of the growth inhibitory influences of aggrecan. Initially, we hypothesized that aggrecan could inhibit axonal growth by influencing Schwann cell migration. If aggrecan decreased Schwann cell migration, this could result in a decrease in possible

axonal promoting molecules or cues, which, in turn could lead to a decrease in axonal growth. However, our results do not support this hypothesis. We did not detect an immunohistochemical pattern that suggests that aggrecan inhibits Schwann cell migration. Furthermore, if aggrecan's ability to inhibit growth was dependent upon its putative influences upon Schwann cells, this would not account the neurite growth inhibitory influences of aggrecan *in vitro* assay where Schwann cells are not present (Snow, Letourneau, 1992). Thus, it seems likely that aggrecan may inhibit growth in other ways.

Aggrecan may inhibit growth by binding with a yet-to-be-identified receptor, that causes events that result in growth rate reduction or inhibition. This seems to be plausible for another specific CSPG, NG2 (Dou, Levine, 1997). It is also likely that aggrecan (or aggrecan CP) may mask the growth promoting sites (such as an RGD sequence) of growth permissive molecules, such as laminin, that is responsible for interacting with neurons and promote neuritic growth. Perhaps aggrecan (or aggrecan CP) blocks the growth promoting receptors, such as integrin receptors (McKeon et al., 1995). In general, CSPGs can bind other ECM molecules, such as tenascin-R (Aspberg et al., 1997) and growth factors, including transforming growth factor-beta (Yamaguchi et al., 1990) through their protein core. These interactions with other molecules could influence growth.

Our results suggest that primary inhibitory component of aggrecan lies in the CP. However the effect of aggrecan CP was not equal to intact aggrecan. Thus, it seems plausible that CS GAG chains of aggrecan may also contribute to its growth inhibitory influences. Although our results show that CS GAG chains can influence growth (Box B), it was not as robust or as widespread as expected. Perhaps aggrecan's inhibitory

influences are derived from both the CP and its CS GAG chains. In particular, the key to aggrecan's influence may lie in its conformation. The ability of the CP to hold the CS GAG chains in a three dimensional brush-like array may result in a steric hinderance against axonal growth and/or influence the way in which a neuronal process may "see" and thus be affected by aggrecan. Perhaps intact aggrecan's presentation to a neuron results in growth inhibition by drawing upon the combined influences of the CP, CS GAG chains and three dimensional conformation of these subcomponents.

In summary, the *in vivo* growth assay can test the ability of distinct CSPGs to influence neurite growth. Our results showed that aggrecan can inhibit neurite growth. In addition, we have shown that CP of aggrecan is capable of inhibiting growth. The influence of CS GAG chains were only seen in Box B but not found in Box A or Box (A+B). Neither the CP or the CS GAG could inhibit growth more so than intact aggrecan. Thus, intact aggrecan appears to inhibit neurite growth *in vivo* and chondroitinase ABC digestion may not sufficiently diminish its effects in this assay.

CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

In attempt to better understand the lack of regeneration following mammalian spinal cord injury, we have begun to examine some of the molecules in the extracellular milieu that may inhibit axonal growth. We have found that the spatial and temporal immunoexpression patterns of chondroitin-6-sulfated proteoglycans (CSPGs) are correlated with a lack of regeneration. Our results show CSPG-immunoreactivity (IR) increases as early as four days post- injury and persists for at least forty days (the earliest and latest time point evaluated). In addition, CSPG-IR remains elevated in the host following intraspinal transplantation and develops within the graft overtime. Interestingly, CSGP-IR was co-localized with glial-fibrillary associated protein (GFAP) in the injured and transplanted spinal cords, suggesting that astrocytes may secrete CSPGs. The spatial and temporal IR patterns of CSPGs suggest that this group of putative inhibitory molecules may contribute to the lack of regeneration in the injured, adult spinal cord.

These correlative studies led to experiments that were designed to more directly evaluate the influences of CSPGs upon axonal growth *in vivo*. At the time these experiments began, recent literature had suggested that distinct CSPG members may have different influences upon growth. Thus, in order to more clearly evaluate if CSPGs could inhibit growth *in vivo*, it is necessary to evaluate CSPG members individually. We focused upon a specific CSPG, aggrecan.

Western blot analysis and aggrecan specific antibodies show that full length aggrecan and aggrecan degraditive fragments are present in the embryonic, early postnatal, adult and injured spinal cord. Furthermore, astrocytes derived from adult brain and spinal cord can secrete aggrecan *in vitro*. These results suggest that aggrecan may be one of the CSPGs that was co-localized with GFAP-IR in injured and transplanted spinal cords mentioned above. It was somewhat surprising to identify such a large proteoglycan in the spinal cord due to the limited space in the extracellular matrix. In cartilage, aggrecan has a large core protein (350kD) and over 100 CS glycosaminoglycan (GAG) chains. This large molecule appears to have a bristle brush like structure in cartilaginous tissue and can form macromolecular aggregates by binding to hyaluronic acid. It is possible that aggrecan in the central nervous system (CNS) may not exist in the same form as it does in cartilage. In the CNS, aggrecan could be smaller and have fewer and/or shorter GAG chains. Based upon the documentation of other similar aggregating proteoglycans in the extracellular matrix of the CNS, it seems likely that aggrecan would also exist in this tissue. However, this can be resolved in the future with aggrecan-specific antibodies that work using immunocytochemistry techniques.

Initially, there appears to be some discrepancies between the CSPG studies and the aggrecan studies. First, in Chapter 2, we show very low CSPG-IR patterns in the E14 spinal cord compared to the adult and injured adult spinal cord. However, in Chapter 3, aggrecan is detected at similar levels in E14 and adult and injured adult spinal cord. These apparent conflicts are most likely to stem from methodological differences. In Chapter 2, an antibody against chondroitin-6-sulfate proteoglycan was used in immunocytochemistry whereas in Chapter 3 antibodies against aggrecan core protein were used in Western blot

analysis. The differences in these antibodies and techniques can contribute to these apparent discrepancies. For example, during development in cartilage, aggrecan does not have many (if any) chondroitin-6-sulfated GAG chains; the majority of GAG chains are chondroitin-4-sulfate. In the adult, however, aggrecan does have more chondroitin-6-sulfated GAG chains bound to its core protein. Thus, the chondroitin-6-sulfate antibodies used in Chapter 2 may not label many of the aggrecan molecules during development. In contrast, the antibodies against aggrecan core protein used in Chapter 3 can label aggrecan equally well in the embryo, the adult and injured adult. Thus, these differences in antibodies can contribute to the results in Chapters 2 and 3 that initially appear discrepant. A second set of seemingly conflicting results is the documented increase in CSPG-IR following spinal cord injury in Chapter 2 and the lack of detection of an increase in aggrecan expression following injury in Chapter 3. This apparent discrepancy may stem from methodological differences also. In Chapter 2, CSPG-IR was examined at the lesion epicenter using immunohistochemistry. In Chapter 3, aggrecan was analyzed in 5mm pieces at and adjacent to the lesion epicenter using Western blot analysis. It is possible that the increase in CSPG-IR and aggrecan expression may be spatially limited to the lesion epicenter. If this is the case, an increase in aggrecan expression may be diluted below our detection levels in a 5mm section of spinal cord tissue that would contain the lesion epicenter and surrounding tissue. In addition, it was difficult to assess differences in aggrecan expression in Western blot analysis due to the multiple banding patterns seen with ATEGQV and THYKRL antibodies. These aggrecan antibodies used in Chapter 3 can label full length aggrecan and aggrecan degraditive fragments. Thus, analyzing differences in overall aggrecan expression from several aggrecan positive bands is difficult.

Also, the composition of CS GAG chains bound to aggrecan following injury are not currently known. It is possible that the sulfation patterns and the length of CS GAGs may change following spinal cord injury. These variables could contribute to the differences seen in CSPG-IR versus aggrecan expression in the injured spinal cord that are described in Chapters 2 and 3, respectively.

Influences of aggrecan upon axonal growth were examined in a novel *in vivo* growth assay. Our results suggest that aggrecan can inhibit axonal growth in this model. One of the most elegant findings that strongly suggest aggrecan to inhibit axonal growth is the presence of swollen end bulbs or terminal clubs at seven days post-surgery in animals with aggrecan loaded-matrices (Figure 5-1, A). Furthermore, it appears that aggrecan and the core protein (CP) of aggrecan can significantly inhibit axonal growth, however CS GAG chains of aggrecan do not appear to consistently inhibit growth at fourteen days post-surgery. These studies are the first of its kind to analyze the effects of a specific CSPG and two of its subcomponents *in vivo*.

The *in vivo* growth assay experiments described in Chapter 4 were designed to examine if aggrecan could influence axonal growth of adult axons. In order to accomplish this task, a robust growth arena was needed to serve as a positive control and to compare to the growth patterns into three experimental groups. Initially, studies examined axonal growth into matrigel-filled hemisections without placement of severed dorsal rootlets. These experiments revealed that some lightly scattered fibers were present in matrigel-loaded matrices without dorsal rootlet placement at two weeks post-surgery (Figure 5-1,B). These fibers were not as abundant as those seen in time-matched matrigel-loaded matrices with dorsal rootlet placement (Figure 5-1,C). Therefore, animals with dorsal

rootlet placement were selected as positive controls and the group of animals without dorsal rootlet placement was not expanded nor quantified for this study. However, the presence of fibers in matrices without dorsal rootlet placement suggests that fibers are capable of extending into matrigel at two weeks without the addition of a dorsal rootlet.

The origin of the fibers in various matrices is not clear. The results from the group of animals without dorsal rootlet placement suggests that not all fibers in the positive controls originate from the grafted dorsal rootlets. Immunohistochemistry techniques can not identify all of the potential fiber types that may or may not enter the matrices because antibodies are not available against all fiber types. In the future, tracing techniques could determine the origin of fibers that do or do not extend into various matrices. However, the origin of fibers was not the focus of the experiments described in Chapter 4. These purpose of the studies in this dissertation was to initially demonstrate that aggrecan can influence axonal growth of adult axons *in vivo*. Future studies could be done, including tracing studies, that could provide information about the possible effect(s) of aggrecan upon different fiber types.

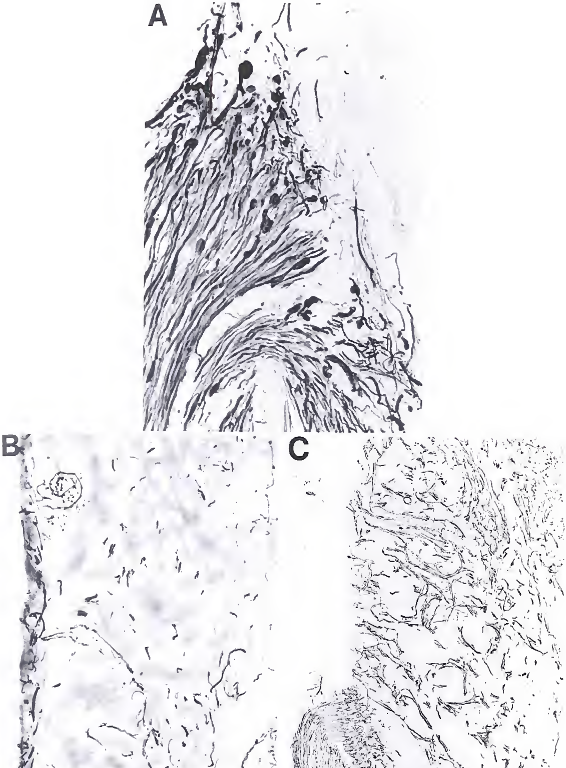
There are several logical extensions to these studies. The influences of other distinct CSPGs (brevican, neurocan, versican etc..) or other growth influential molecules could be tested in this *in vivo* assay. The future studies that are described below are described relative to aggrecan but can also be applied to other growth influential molecules as well.

The *in vivo* growth assay has the potential to identify potential part(s) of the CP of aggrecan, is(are) capable of inhibiting axonal growth. Future studies could examine the effects of particular regions of the CP, such as the G1 domain, upon axonal growth. Furthermore, the influences of aggrecan degradation fragments that are present in the

spinal cord, such as the 220kD TKKEEE positive band, could be also evaluated in this assay. These experiments could provide information that could help clarify aggrecan's mechanism(s) of growth inhibition.

The influences of aggrecan upon Schwann cell migration can be clarified in cell culture assays. The influence of aggrecan upon Schwann cell migration could be evaluated with immobilized and/or soluble aggrecan *in vitro*. Furthermore, the influence of Schwann cells upon axonal growth in the *in vivo* growth assay may be addressed by seeding Schwann cells into aggrecan-loaded matrices prior to placement into the hemisection lesion. If axonal growth into the Schwann cell seeded, aggrecan-loaded matrices is equally inhibited as the aggrecan-loaded matrices alone, this could suggest that aggrecan may inhibit axonal growth independent of the presence of Schwann cells. Otherwise, if axonal growth is less inhibited into Schwann cell-seeded, aggrecan-loaded matrices as aggrecan loaded-matrices, this could suggest that Schwann cells may be able to guide axons into the matrix despite the presence of aggrecan.

Figure 5-1. Neurofilament positive staining labels neuronal cell bodies and processes in representative photomicrographs of hemisected spinal cords filled with various matrices. At seven days post-surgery, swollen end bulbs are seen at a high magnification on dorsal rootlet fibers that were placed into aggrecan-loaded matrices (A). At fourteen days post-surgery, fibers are lightly scattered in matrigel-loaded matrices without dorsal rootlet placement (B) compared to the abundance of fibers in timed-matched matrigel-loaded matrices with dorsal rootlet placement (C).


A
B
C

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BIOGRAPHICAL SKETCH

Michele Lynn Lemons was born in Bethesda, Maryland, on May 21, 1971. Michele obtained her primary education in Herndon, Virginia, at a small Catholic school, St. Joseph's, until the 8th grade and graduated from Herndon High School four years later. She obtained her bachelor of science degree and a certification in secondary education from The College of William and Mary in Williamsburg, Virginia, in May of 1993. Michele's interest in the field of neuroscience evolved from the courses she took as a biology major and psychology minor at William and Mary. Her interests in science were strengthened upon learning of her mother's diagnosis with myasthenia gravis. Michele began graduate school in the department of neuroscience at the University of Florida in the summer of 1993. After rotating through three laboratories, Michele decided to work in the laboratory of Douglas K. Anderson for her dissertation project. She worked very closely throughout her graduate education with another scientist, Dena R. Howland.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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